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TABLE OF CONTENTS.

Ross G. Harrison. Some unexpected results of the heteroplastic transplantation of limbs. *Proceedings of the National Academy of Sciences.* Vol. 10, February, 1924.

J. S. Nicholas. Ventral and dorsal implantations of the limb bud in Amblystoma punctatum. *Journal of Experimental Zoology.* Vol. 39, February, 1924.

William E. Greenleaf. The influence of volume of culture medium and cell proximity on the rate of reproduction of Protozoa. *Proceedings of the Society for Experimental Biology and Medicine.* Vol. 21, April, 1924.

E. Lucile Moore. Regeneration at various phases in the life-history of the infusorians Spathidium spathula and Blepharisma undulans. *Journal of Experimental Zoology.* Vol. 39, April, 1924.

Lorande Loss Woodruff and Hope Spencer. Studies on Spathidium spathula. II. The significance of conjugation. *Journal of Experimental Zoology.* Vol. 39, April, 1924.

George A. Baitsell. Observations on the cellular activity in a culture of amphibian liver tissue. *Proceedings of the Society for Experimental Biology and Medicine.* Vol. 21, May, 1924.

W. W. Swingle and J. S. Nicholas. Autoplastic and homoplastic parathyroid transplantation. *Proceedings of the Society for Experimental Biology and Medicine.* Vol. 21, May, 1924.

Lorande Loss Woodruff and E. Lucille Moore. On the longevity of Spathidium spathula without endomixis or conjugation. *Proceedings of the National Academy of Sciences.* Vol. 10, May, 1924.

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Rhoda A. Hartwell. A study of the olfactory sense of termites. *Annals of the Entomological Society of America.* Vol. 17, June, 1924.

Lorande Loss Woodruff and W. W. Swingle. The effects of thyroid and some other endocrine products on Paramecium. *American Journal of Physiology.*. Vol. 69, June, 1924.

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A. Elizabeth Adams. An experimental study of the development of the mouth in the amphibian embryo. *Journal of Experimental Zoology.* Vol. 40, October, 1924.

W. W. Swingle. Is the tadpole test for thyroid valid? *Endocrinology.* Vol. 8, November, 1924.

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SOME UNEXPECTED RESULTS OF THE HETEROPLASTIC TRANSPLANTATION OF LIMBS

By Ross G. Harrison

Osborn Zoölogical Laboratory, Yale University

Read before the Academy, Nov. 12, 1923

The embryos of distinct but closely related species of amphibians often differ from one another in certain qualities, such as pigmentation, size, time of appearance of organs, etc., which render them useful in the analysis of the factors of growth and development by means of grafting experiments. Two of our native salamanders, Ambylstoma punctatum Linn. and A. tigrinum Green, afford advantages of this kind in connection with the development of the limbs.

In the spotted salamander, A. punctatum, the fore legs begin to show externally at a comparatively early stage and but little later than the external gills. By the time the yolk is resorbed, they are fully developed both in form and in function. On the other hand, in the tiger salamander (A. tigrinum) the fore limbs at this period are small nodules still, scarcely visible superficially. It is not until some weeks later that they reach the degree of development attained by the fore limbs of the punctatum larva at the close of the embryonic period. With respect to the development of most of the other organs the embryos of these two species show much greater concurrence, but there are several characteristic differences that require brief notice here.

The tigrinum egg is smaller and contains much less yolk, so that this store of food is exhausted sooner than in the punctatum egg. The tigrinum larva consequently begins to feed when quite small, although, except for the difference in the fore limbs, the embryos of the two species are approximately at the same stage of development at this period. During the first part of larval life the rate of growth is not very different in the two species, but after five or six weeks, when they have reached a length of about 4 cm., the tigrinum larvae begin to forge ahead rapidly and ultimately attain a much larger size than the punctatum. Sexually mature larvae (axolotls) that have come under my notice measure from 25 to 32.5 cm. in length. The two longest adult specimens mentioned by Cope[1] measured 25 and 27.5 cm., respectively. In the strain used in the present investigation metamorphosis has not been observed to take place in specimens less than 13 cm. in length, and it often, apparently, does not occur at all, while in A. punctatum it takes place when the larvae are about 5 cm. long. This size may be reached under favorable circumstances in the latter species in about ten or twelve weeks, at which time tigrinum larvae of the same age, kept under similar conditions, are about 6 cm. long,

without, however, showing any sign of metamorphosis. Adult punctatum individuals measure about 16 cm. in length.

For the purpose of investigating the factors concerned in the development and growth of the fore limbs, limb buds were interchanged between embryos of the two species. The grafting was done in the usual way at an early period of development (stages 29–31),[2] before the limb buds could be distinguished as such, and before peripheral nerves or blood vessels were present.

The initial effect of the operation was as expected, especially in the one series of experiments. The punctatum limb implanted on a tigrinum embryo grows rapidly, and is soon far beyond the normal tigrinum limb in development. It does not, however, keep pace with the normal undisturbed punctatum limb of the donor, and it does not during this period acquire its normal powers of movement.

In the reciprocal experiment the tigrinum limb on the punctatum embryo remains very small for a short time, while the normal limb on the opposite side grows out rapidly. In this case, however, the effect of the transplantation soon becomes evident in a positive manner, for the grafted limb begins to grow far more rapidy than it would have, had it been left in place. When the punctatum larva begins to feed (stage 46) the normal limb has three digits with the beginning of the fourth, and it functions perfectly. While the grafted tigrinum limb is less advanced than this, it has, nevertheless, lengthened far beyond the normal tigrinum limb, which is still a nodule, and it shows the notch marking the first two digits, a condition reached by the normal punctatum limb in stage 42. Such grafted limbs even show slight movements at the shoulder, although the normal punctatum limb does not reach this functional condition until stage 44, when the hand is much better developed.

The subsequent behavior of the grafted limbs in both series of experiments is notable for the increasing influence of the host on their growth.

The transplanted punctatum leg lags more and more behind the normal fore leg of the donor. For a time it appears atrophic, and its only movement is a slight twitching at the shoulder. This lack of motility is in itself interesting, for sections of individuals preserved during this period show that the nerves of the limb are well-developed. The absence of normal function is, therefore, probably due to conditions within the central nervous system, corresponding to the rudimentary state of the normal tigrinum limb. In several cases the transplanted leg seemed for a time to be undergoing degeneration. At the same time the tigrinum host grew rapidly, and its own fore leg, in the course of about six or seven weeks from the date of operation, overtook and soon exceeded in size the grafted one (figs. 1 and 2), which remained very small, though it later acquired perfect form and function, all traces of degeneration disappearing. Three such

specimens have been kept through this stage. One (fig. 1) was preserved and sectioned, showing a diminutive shoulder girdle as well as limb. Two are still alive. One of the latter metamorphosed when 13 cm. long, at which time the grafted limb was resorbed to a short stump. The other, now 14.7 cm. long is still a larva. The transplanted limb is very small as compared with the normal limb of the host, but it is now larger than the limb of the punctatum donor, which latter is a much smaller animal, 6.6 cm. long. The function of the grafted limb is perfect. Its shape is that of the donor species (punctatum), but the skin markings and coloration are those of A. tigrinum, showing that extensive pigment migration must have occurred.

Fig. 1.—Larva of Amblystoma tigrinum with a punctatum fore limb (*gr*) on the right side; *n*, normal limb. Exp. NE.10; specimen preserved 76 days after operation.

Fig. 2.—Larva of A. punctatum, the donor of the limb shown in fig. 1. Regeneration of the lost limb has not occurred. Specimen preserved 76 days after operation.

Fig. 3.—Larva of A. punctatum, showing a gigantic, but otherwise normal, tigrinum limb (*gr*), grown to this size after having been grafted in the limb bud stage; *n*, normal limb. Exp. NE.13, specimen preserved 72 days after operation.

Fig. 4.—Normal control larva of A. tigrinum of the same age.

All figures magnified 1.4 diameters.

The tigrinum limb grafted to the punctatum larva grows very rapidly after it once begins, so that shortly after the yolk is resorbed it overtakes the normal limb in degree of development, at the same time having become

more massive. It is at first white and almost without pigment cells, like the developing tigrinum leg, whereas the punctatum leg is well-pigmented. The grafted limb soon outstrips the normal one on the opposite side, growing rapidly until the time of metamorphosis, when in the most extreme case its linear dimensions are fully double those of the normal (figs. 3 and 4). These animals are very grotesque in appearance, especially when viewed from the front. The gigantic limbs, however, like the diminutive ones in the reciprocal experiment, are well proportioned and function perfectly. The shoulder girdle and pectoral muscles are likewise enormously developed for the size of the animal. None of the five specimens of this series survived metamorphosis.

Sections show that the constituent parts of the limbs in both kinds of transplantations are proportionately developed. Skeleton, muscles, and nerves are small in the miniature transplanted punctatum limb and large in the giant tigrinum limb on the punctatum larva. However, neither the central nervous system nor the spinal ganglia show hypoplasia in the one case or hyperplasia in the other. This may seem odd in connection with Detwiler's[3] discovery that dimunition or increase in the peripheral area innervated by a given nerve results, respectively, in the hypoplasia or hyperplasia of the ganglion of that nerve. It should be recalled, however, that the limb does not immediately show its ultimate growth powers after either type of transplantation. On the contrary, the early tendencies are opposite to the later ones in this respect, both kinds of limb buds at first manifesting their own inborn characters, so that the punctatum limb on the tigrinum embryo is for some time actually larger than the normal tigrinum limb, and the grafted tigrinum limb requires about a month to overtake the normal punctatum limb in size. In this early formative period, therefore, the tendency would be toward hyperplasia of the spinal ganglia innervating the limb that ultimately remains smaller, and toward hypoplasia of those innervating the limb that ultimately becomes much larger. Apparently the limb does not reach its ultimate disproportionate relation sufficiently early to produce an effect on the sensory nerve centers.

In order to account for the above results it is necessary to assume that two distinct factors, aside from the ordinary nutritional ones, are concerned in the growth of the limbs. One of these, immanent in the cells of the transplanted limb and derived from the general constitutional qualities of the species, may be termed the growth potential (G). This is much greater in A. tigrinum (t), as shown by the much larger ultimate size of individuals of this species in comparison with A. punctatum (p). The other factor may be designated as a regulator (R), since it acts by controlling the rate of growth. It exists not in the individual cells of the transplanted tissue, but in the circulating medium, and is probably a hormone

derived either from the hypophysis or the thyroid gland. Since A. punctatum metamorphoses very early as compared with A. tigrinum, there is justification for the assumption that this regulating factor is more effective in the former species. The relative effect of the four combinations (two normal and two experimental) of these factors, acting until the time of metamorphosis of the punctatum larvae (ten or twelve weeks after grafting), may be expressed by the inequality

$$G_tR_p > G_tR_t > G_pR_p > G_pR_t$$

What the ultimate relation of these effects would be if the salamanders were kept until they all reached full size remains unknown, because no individuals of the first combination have survived metamorphosis. In other words, we do not know whether the grafted tigrinum leg would ultimately exceed its normal size, or whether it would simply reach this condition in less time and then remain stationary. We do know, however, that the order of the last two terms will be reversed later, for the actual size of the grafted punctatum limb in the oldest specimen under observation is larger than that of the normal control limb of that species. Here, however, there is a likelihood that nutritional factors are concerned, for the tigrinum individual with the grafted leg is very much larger than the punctatum donor, and presumably a larger supply of nutrient material reaches the grafted leg than the leg of the latter.

The foregoing experiments show with great clearness that various influences are involved in growth. However, it is desirable here to emphasize the importance of the constitutional factors, because they are more likely to be overlooked. The grafted limb is subject to exactly the same nutritional and hormonic influences as the normal limb of the same individual, and yet, by virtue of its specific constitution, its growth is enormously greater in the one and correspondingly less in the reciprocal combination than the growth of the normal control limb on the opposite side of the body. Again when a comparison is made between the grafted limb and the control left on the body of the donor, then the importance, in growth, of the regulatory hormone stands out, for the relative acceleration or retardation, as the case may be, is very marked. This too is a constitutional factor, but in a different sense, for the production of the regulator is probably local and its distribution is effected by the circulating medium, whereas the growth potential is a quality of the tissue cells in general.

Experiments to test the hypothesis regarding the source of the regulating factor have been devised and will be carried out as soon as material is available.

Summary.—The fore limb of Amblystoma punctatum develops during the embryonic period, but that of A. tigrinum relatively much later. The

latter, however, preserving its proportion to the body as a whole, attains ultimately a much greater absolute size.

The fore limb bud of A. punctatum, engrafted in normal position on a tigrinum embryo, develops at first much more rapidly than the natural limb on the opposite side of the host, but is retarded in comparison with the limb of the donnor left in place. In the reciprocal transplantation the tigrinum limb bud remains for a time small, but its growth is soon greatly accelerated and the limb later becomes much larger than that of either species in its normal surroundings.

These results may be explained by the assumption that two factors are concerned in the growth process: the growth potential, a property of the cells of the graft; and a regulator, probably an internal secretion of the host, carried to the limb through the circulation.

[1] Cope, E. D. 1889. "The Batrachia of North America." *Bull. U. S. Nat. Mus.*, No. 34.

[2] Harrison, Ross G. 1918. "Experiments on the Development of the Forelimb of Ambylstoma, a Self Differentiating Equipotential System." *J. Exp. Zoöl.*, **25**.

[3] Detwiler, S. R. 1920a. "On the Hyperplasia of Nerve Centers Resulting from Excessive Peripheral Loading." *Proc. Nat. Acad. Sci.*, **6**, pp. 96–101. Also 1920b. "Functional Regulation in Animals with Composite Spinal Cords." *Ibid.*, pp. 695–700.

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VENTRAL AND DORSAL IMPLANTATIONS OF THE LIMB BUD IN AMBLYSTOMA PUNCTATUM[1]

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TWO PLATES (NINE FIGURES)

INTRODUCTION

In accordance with the rules of symmetry, formulated by Harrison ('21), the transplanted fore-limb bud of Amblystoma develops into a limb corresponding to its side of origin (right or left), if its dorsoventral axis is placed in normal orientation with respect to the host and into a reversed limb if this axis is inverted.[2]

If the validity of these rules is general, a limb bud implanted in the dorsal or ventral midline should produce two limbs, and each limb should have the asymmetry corresponding to the side upon which it is developing, provided that the anteroposterior axis of the limb disc is in normal relationship to the anteroposterior axis of the embryo. If the anteroposterior axis is reversed, each limb should be of the side opposite to that on which it is placed. It has already been shown, Harrison ('18), that the limb is an equipotential system and that parts smaller than half of the normal bud will produce a complete limb.

In order to test the above question a large number of grafting experiments have been made, the results of which are reported in the present communication.[3]

Transplantation of the limb bud to the dorsal or ventral midline, as shown by these experiments, does result in the

[1] Anat. Rec., vol. 23, p. 29.

[2] See Harrison ('21), p. 4, for rules of symmetry.

[3] Nicholas ('21), Anat. Rec., vol. 21, p. 74.

development of two complete limbs from a single bud in those cases in which development takes place at all. The limb bud separates itself into two parts, one part giving rise to a right, the other to a left limb.

In addition to the question of symmetry, several interesting points came up in connection with the influence of the transplanted limb upon the developing nervous system.

This problem was undertaken at the suggestion of Professor Harrison to whom I express my thanks for suggestions and criticism.[4]

EXPERIMENTAL

Embryos of Amblystoma punctatum, operated at the standard stage 29,[5] were used throughout these experiments. In all series the transplanted tissue consisted of the right limb bud, a disc of tissue, three and one-half somites in diameter, lying below the phonephros.

In the ventral implantations the embryo which was to receive the transplant was placed upon its dorsal surface in a grooved wax dish. A circular incision, two somites in diameter, was made in the ventral midline about midway between the stomodaeum and proctodaeum, which form very convenient markers.

The incision was made much smaller than the transplant, for in the ventral region there is a rapid expansion of the wound area after the initial incision. The wound thus accommodated itself to the size of the limb bud. If the incision was made the size of the transplant, healing was considerably delayed and the yolk substance exposed for so long a time that disintegration of the yolk mass took place. Healing of the graft was effected by holding the transplant securely in place with glass rods and was usually complete within forty-five minutes.

The ventral implantations were located in the midpoint of the midline in the majority of cases. The limb bud was also placed

[4] The operative work for the experiments here reported was carried out at the Osborn Zoölogical Laboratory during 1918–19. Laboratory facilities were provided here for the study of material during the summer of 1922.

[5] Harrison ('21), p. 8.

anterior and posterior to this location in some, but this change gave no better results than the original location.

An incision was made in the ectoderm over the medulla for the site of the dorsal implantations. A round piece of ectoderm, three somites in diameter, was removed and the limb disc implanted in the wound. Care was taken not to injure the cord and other underlying structures. The limb was transplanted to the head region in several cases, but none of these showed adequate development. This same disappointing result was obtained when the transplant was placed posterior to the 4th ventricle.

Ventral implantations with anteroanterior orientation

After the healing of the wound, there was a period of two or three days in which there was no superficial indication of the limb. Stage 38,[6] which shows the distinct swelling of the limb upon the surface of the body, showed an equal swelling in the region of the ventral implantation.

In many cases, this was the extent of visible growth in the transplanted limb. When development proceeded no further, all superficial trace of the limb was lost. Microscopic examination of sectioned material preserved at a later stage of development showed that while the free extremity failed to grow, the girdle continued to develop for a considerable period though ultimately development ceased and resorption began. A small portion of the pronephros was implanted along with the limb rudiment. This developed in its new location and was one of the last things to be resorbed.

In other cases, the limb elongated slightly before growth ceased. Most of these showed abortive growth of a double blastema, indicating the presence of two potential areas of limb-forming material. When some portion of the free extremity developed, even though this was abortive, there was considerably more of the girdle remaining in the later stages of development than was found when no extremity whatever developed.

[6] Harrison ('18), p. 420, fig. 4.

In one instance only in this series (fig. 1) was there a complete development of two limbs. This individual showed that in cases, in which the double blastema appeared, development of both portions of the blastema was possible.

While growth was noted in the ventral region, it was not until five days after the operation that the double blastema could clearly be recognized. The two limb processes were independent from the very beginning and were directed posteriorly in their growth. In earlier stages of development, these limbs were slightly delayed as compared with the normal limbs but the end result was not retarded. The cleft marking the bifurcation of the first two digits appeared at the same time in both the normal and the operated limbs, but subsequent digitation was slower in the transplanted limbs. Thirty-three days after the operation, both transplanted limbs were completely developed and, while nonfunctional, were sensitive to mechanical stimuli.

The palmar surface was ventral in these limbs (fig. 1), i.e., it was turned away from the body wall, whereas in its normal position the palmar surface faces the lateral body wall. This result was due mainly to the supinated condition of the limb and secondarily to the mechanical effect produced by the weight of the animal which reduced the slight pronation, which was present before the balancers were lost. The limbs at that period of development had their palmar surfaces turned slightly toward each other but they became flattened shortly after the loss of the balancers. The divergence of the two limbs was exaggerated by this supination and the distance between the heads of the humeri was increased by development of the scapula rudiment. In the pronated condition found in normal limbs, the ulna and radius are crossed. In the present case, the radius and ulna were parallel throughout their extent.

Examination of sections through the region of the transplanted limbs showed that a complete double girdle had been formed (fig. 5). The two girdles were fused in the midline and there were two complete and separate glenoid cavities, with each of which a single humerus articulated. The girdles were practically symmetrical and extended dorsally and laterally along each

flank. In the region mesial to the glenoid cavities there was found a muscle mass which was common to both of the limbs. The muscles lateral to the glenoid cavities were complete for each member, although they were smaller than those usually found in connection with a transplanted limb. The nervous supply was derived from the nerves running to the ventrolateral musculature. The nerves were smaller than those ordinarily found in the limb and could not be followed to their distal extremities. The nerve cells of the ganglia corresponding to the innervated segments have been counted but there was no evidence of the hyperplasia which Detwiler ('20) found to occur as a result of overloading the peripheral nervous system.

TABLE 1

SERIES	NUMBER OF OPERATIONS	CONDITION OF THE LIMBS			SHOWED DIRECTION OF GROWTH DURING DEVELOPMENT	DISCARDED[2]
		Single	Double	Absorbed		
RA[1]	76	4	1	50	27	21
RB	60	4 Abort.		40	38	16
RC	22	2 Abort.	1	14	12	6
RD	18	3 Abort.		11	10	4

[1] Series RA, ventral implantations with anteroanterior orientation; RB, with posteroanterior orientation. Series RC, dorsal implantations with anteroanterior orientation; RD, with posteroanterior orientation.

[2] Under the heading of discarded animals are included all deaths and all animals whose case records showed no superficial appearance of the limb blastema.

The limbs were non-functional but this could be accounted for by the absence of nerves from the normal limb level (Detwiler, '22). A common muscle mass was attached to the mesial surface of each humerus. This would tend to limit lateral arm movements. The limbs were sensitive when touched lightly with a hair. Sensory innervation was, therefore, probably present.

Ventral implantation with posteroanterior orientation

While no case of complete development occurred in this series, the evidence which was given by a study of the early development of the transplant was interesting. It showed that

if the conditions necessary for complete development had been found in this series, two limbs with asymmetry opposite to the side on which they were placed would have developed, i.e., the limb on the left side would be a right and that on the right side a left. This tendency could be seen in the early development of the limb disc before resorption began.

The blastema of the limb with its anteroposterior axis reversed appeared about one full day later than the blastema of the preceding series. A large nodule was formed from this blastema and, as in the preceding series, this was often the only growth which took place. Most of the cases showed a division of the blastema shortly after its appearance, but the whole divided blastema disintegrated without further development. When the limb bud developed a free extremity, this was always directed toward the head and was found on either one side of the body or the other since in no case was there a complete double development. The asymmetry of the free extremity could not be determined in the cases tabulated above as abortive. All showed initial doubling, but one of the components was resorbed. The remaining portion was also abortive due to the same condition.

The girdle structures remaining after the resorption of the free extremity indicated the doubling that had taken place and all of the girdles in which the asymmetry could be determined were reversed.

The points taken as criteria for the orientation of the girdle are as follows: 1) the location of the glenoid cavity, which is found at the posterior portion of the implanted girdle in all animals in which the transplant has been oriented antero-anteriorly, and is anterior in those with reversed asymmetry; 2) the position of the small scapular rudiment, which in the orthotopic location would unite with the suprascapula. This is anterior in girdles with normal asymmetry and posterior in those which are reversed.

The coracoid and procoracoid which serve as criteria in transplantations upon the flank are not definite enough in the dorsal and ventral regions. They are similar to each other in size and

shape and can only be taken as criteria after determining their relationships to the structures described above.

A large number of the girdles, however, were anomalous and had lost their symmetrical relationships by the resorption of the outlying parts which were used in orientation. Many of the girdles consisted of a simple rounded plate of tissue.

Dorsal implantation with anteroanterior orientation

In the one case (fig. 2) in which limbs developed typically, two perfect limbs were present, the one upon the right side being a right limb while the other was a left. These limbs were morphologically complete except that motor nerves could not be traced into them. There was a double girdle present (fig. 6) the central portion of which was common to both limbs. Two humeri were found and the digits and other parts of the limbs were approximately normal.

There was no other case of typical development, most of the limbs being resorbed, while those that survived this process were abortive and irregular (figs. 3 and 4). Sectioned material from these animals (fig. 9) showed that the girdles developed in the absence of the limb. Such girdles were generally doubled.

Sections of the animal in which the limbs developed, showed that there was an enlargement of the nervous system in the region ventral to the transplant (fig. 7). The cells of the mantle layer outgrew the marginal layer, which only partially enveloped the cord. Numerous mitoses were found in the cells making up the region of enlargement. The sensory ganglia in the region of the transplant were displaced from their normal location and have been drawn upward so as to lie dorsal to the cord (fig. 8). Fibers from these ganglia have been traced into the limbs. It was impossible to follow the motor fibers in their entirety, and although the direction of the fibers seemed to indicate that they might have gone into the transplanted limbs, most of them were lost in the mesenchyme at the base of the limbs.

Dorsal implantations with posteroanterior orientation

The results of these experiments were negative except for the evidence of reversal afforded by the study of the girdles. The direction of the limb growth was toward the head and the orientation of the girdles was reversed, with respect to the body as a whole, in those cases in which criteria for determining the asymmetry remained.

DISCUSSION

Symmetry

According to an extension of the rules of symmetry, a symmetrical pair of limbs should develop in midline transplantations. This is the case in those experiments in which limb development took place. Moreover, a study of the early development of the transplants showed that the blastema, in many cases, was a bilateral structure and showed a direction of growth which corresponded to that which was expected from the orientation given to the limb disc.

When development did not progress sufficiently to determine the asymmetry by means of the free extremity, the girdle often gave an important clue. In all cases in which the asymmetry of the girdle components could be determined, the portion of the girdle upon the left side of the animal was a left girdle while the right portion was a right, when the limb disc was oriented in normal relationship to the anteroposterior axis of the embryo. When this relationship was reversed, the girdle components were also reversed. The left girdle was then upon the right side while the right girdle was upon the left. Limbs associated with such girdles were directed toward the head in their growth.

Wilhelmi ('21) transplanted two left limbs to the ventral midline in Triton embryos, orientating them so that the dorsal portion of the transplant was anterior with respect to the embryo. This is equivalent to a 90° rotation. The transplants were placed one behind the other presumably far enough to prevent developmental influences of one upon the other. From this

experiment she secured one case in which two left limbs were produced, one from each transplant.

In a subsequent publication Wilhelmi ('22) has performed experiments in the ventral midline with both right and left limb buds. In no case did she secure the formation of two complete limbs from a single limb bud. She has used the fore- and hind-limb buds in these experiments, some of which are more nearly comparable to my own than the case first cited. Such cases appear in her Abb. 9 and 10 in which she has reversed the anteroposterior axis of one of the transplants. In both cases these gave rise to single limbs.

While the number of cases in the present investigation is much too small to permit generalization, yet it seems as though the results point to the potency of each half of the limb bud to produce a limb[7] when placed in the ventral or dorsal midline. Wilhelmi secured a similar result which is shown in her Abb. 24, S. 214, in which one component has been completely resorbed. She does not seem to regard this as the typical result of such transplants.

Resorption

Resorption of the limb graft has been noted in previous works upon the limb. Braus has found it in the Anura and Harrison has noted it in certain regions of Amblystoma. Detwiler ('22), has shown the abnormalities which may appear in the limb due to the implantation in the active gill forming region. The failure of the limb to grow properly, except in Detwiler's cases where there is a specific tissue dominance, was due probably to faulty blood supply. At times, the vessels fail to enter the limb at all, at others the supply is not sufficient to carry on the needs of the developing structure. In general, the further the limb is displaced in either the dorsal or ventral directions from its normal location, the less likelihood is there of adequate circulation.

[7] Harrison ('21), pp. 69–78.

Effect upon the nervous system

So far as can be ascertained by counting the cells of the ganglia and the cord, there was no effect on the nervous system from the peripheral overload brought about by the developing ventral limbs. These limbs contained nerves which can be found in section. Innervation was accomplished by the ingrowth of nerves which go to the ventrolateral musculature.

In the dorsal implantations, however, injuries to the cord during the operation or pressure upon it due to the development of contiguous structures might be expected to produce changes. Development of a graft follows a regular sequence; the double girdle is formed above and outside of the spinal cord and has no effect upon it in those cases in which the girdle parts have developed while the limb has failed to develop.

In the one case in which the double limb developed fully, the picture is radically different. The cells of the cord are in an active condition of mitosis. The dorsal root fibers pass dorsally from the cord to join the ganglia, and fibers can be traced through the ganglia to the transplanted limbs.

While the conditions found in the cord can be ascribed to the development of foreign structures which have injured the normal relationships there, this will not account for the displacement of the ganglia and the distribution of the fibers from the displaced ganglia to the transplanted limbs. This could result only from an attractive force which possessed a definite affinity for nervous structures.

Whether this is neurobiotaxis in Kapper's sense is beside the immediate question. It certainly shows that a developing limb can exercise a positive attractive force upon the outgrowing nerve fibers in its proximity. This force deflects them from their normal lines of outgrowth and causes them to follow an entirely new course and to innervate a region which is entirely foreign to their normal distribution.

SUMMARY

1. A limb bud transplanted to the dorsal or ventral midline has the potency for the production of two limbs. The two limbs are symmetrical with each other and each corresponds to the side upon which it develops when transplanted with normal relationship to the anteroposterior axis of the body. It is reversed in asymmetry when the relationship is reversed. This is shown by the relations of the girdle parts remaining after the resorption of the limb.

2. The dorsal and ventral midlines are distinctly unfavorable locations for the limb. In the majority of cases development of the limb is initiated in the new location, but complete development does not take place.

3. When the development proceeds, the limbs are topographically complete in every particular and contain nerves which are derived from the nearest nerve trunks. The ventrally implanted limbs get their innervation from nerve trunks which go to the ventrolateral musculature, while the dorsally implanted limbs secure innervation from the segment of the cord in proximity to them.

4. No hyperplasia of the sensory or motor elements of the cord has been noted in connection with the ventral implantations. In the case of the dorsal transplantations, however, there is hyperplasia of the dorsal region of the cord. This hyperplasia is not found in any animals in which there was incomplete limb development.

5. Resorption is the greatest factor in the failure of the limb to grow properly. This is probably caused by lack of nutrition resulting from an inadequate blood supply.

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PLATE 1

EXPLANATION OF FIGURES

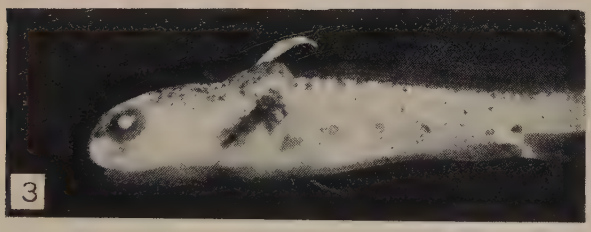
1 This figure shows the development attained by the limb in the ventral location. This is the only case in which development was completed. The palmar surface of the transplanted limbs is toward the reader. The section shown in figure 5 was taken through the base of the limbs shown in this figure.

2 The dorsally developing limbs have their extensor surfaces toward the reader in this figure. The limbs have been slightly displaced toward one side during their growth. For figures of the girdle and also of the underlying cord see figures 6, 7, and 8.

3 and 4 Show the less complete development which was generally realized after transplantation to the dorsal midline. Figure 9 is a section taken from an animal in which resorption had proceeded further than shown in figure 3.

1

2


3

4

PLATE 2

EXPLANATION OF FIGURES

All figures were taken from sections stained with Mallory's connective stain and cut 10μ. Figures 5, 6, and 9 were photographed at a magnification of 50 diameters, the right side of the section appears as the left. Figures 7 and 8 were photographed at 100 diameters, with compensationocular no. 4.

ABBREVIATIONS

G, ganglion
GR, glenoid region of scapula
H, section through the humerus
M, cells of the mantle layer of the cord
P, pronephros
PC, procoracoid
PF, pronephric funnel
S, scapula
SS, suprascapula

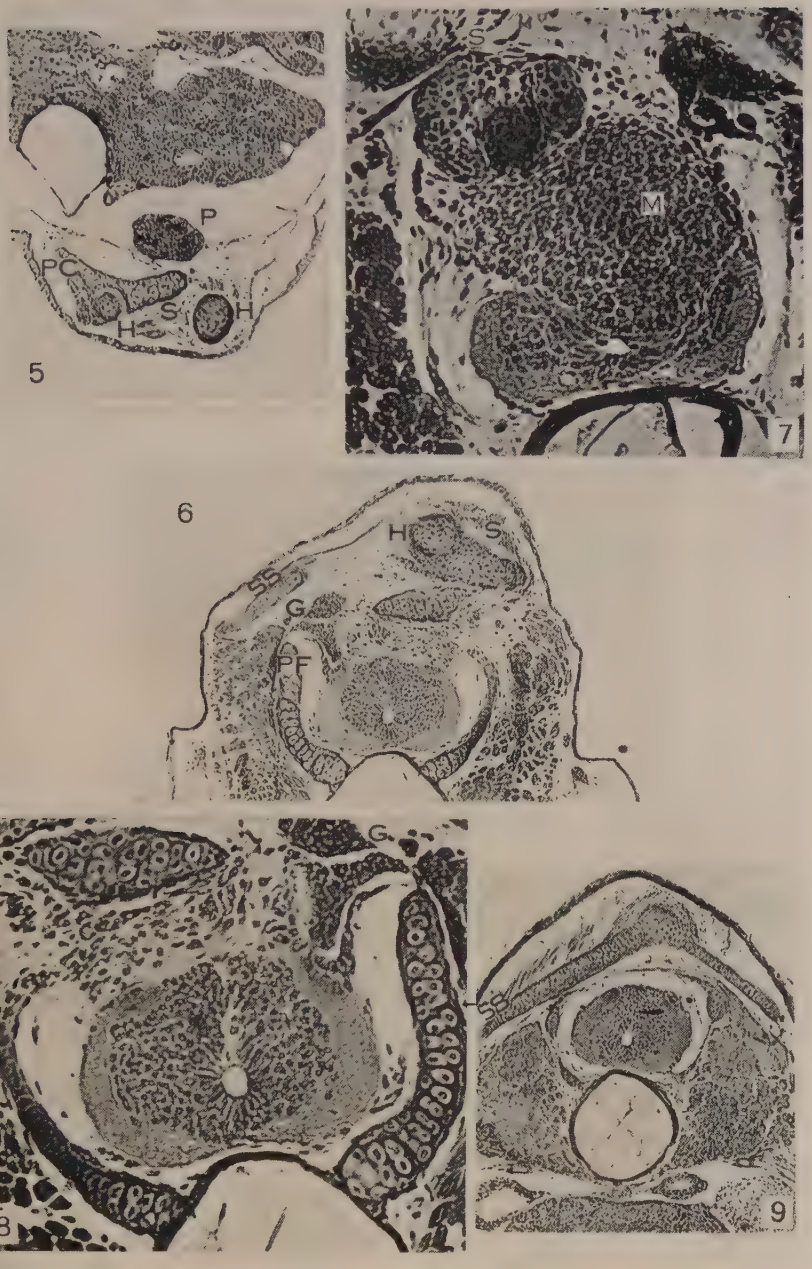
5 Section through the double girdle of the animal shown in figure 1. The two humeri are visible and also the glenoidal articulation of the right limb. The pronephric rudiment (P) is shown dorsal to the girdle. This is outside of the cavity and is covered by a thin layer of peritoneum. × 50.

6 A section taken from the base of the limbs of the animal shown in figure 2, to show the relationship of the developing structures. × 50.

7 Section through the cord underlying the transplanted dorsal limbs. This section is slightly posterior to that shown in figures 6 and 8. There are active mitoses in this region and the cell ratio in comparison to segments of the cord slightly anterior to the limbs is 5 to 1. × 100.

8 An enlargement of the figure 6 to show the fibers entering the dorsal root ganglion. It will be noted that the ganglion is dorsal to its normal location. The ganglion of the opposite side does not show in this section.

9 A section through the region of implantation of the limb which was resorbed early. The girdle is fully formed although limb development never took place. It is practically symmetrical. The central portion, marked *GR*, glenoid region, which ordinarily forms the glenoid cavity is a knob shaped mass of cartilage.


P
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203 (2435)

The influence of volume of culture medium and cell proximity on the rate of reproduction of Protozoa.

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Experiments were undertaken to determine (1) the relationship between the volume of culture fluid and the division rate of Infusoria, and (2) the effect of cell proximity on the division rate. The Infusoria studied were pedigree races of *Paramecium aurelia, Paramecium caudatum,* and *Pleurotricha lanceolata.* The experiments comprised four lines of each species bred in 2, 5, 20 and 40 drops of hay infusion. Forty drops were approximately equal to 1 cc. At the end of five day periods the average per diem division rate was obtained for the four lines in each volume.

In the set of experiments with different volumes of culture medium, the average per diem rate was higher in the larger volumes, as shown by the following data, based on the averages for twenty-seven experiments lasting through a period of five days each for the three species mentioned, or nine experiments per species: animals in 2 drops, 0.92 division; in 5 drops, 1.03 divisions; and in 20 drops, 1.20 divisions. Eighteen of the experiments, or six experiments per species, included four lines each in 40 drops with an average per diem division rate of 1.27. Woodruff[1] in his studies on the effect of excretion products of

[1] Woodruff, L. L., *J. Exp. Zoöl.*, 1911, x, 557; *J. Exp. Zoöl.*, 1913, xiv, 575.

Protozoa on their rate of reproduction obtained equivalent results and interpreted the lower division rate in smaller volumes of medium as due to the effect of the excretion products of the animals.

The second set of experiments differed from the above in that it was confined to a pedigree race of an hypotrichous infusorian and, instead of one animal, two animals were bred together on each slide, in order to determine whether, as Robertson[2] maintained in his studies on *Enchelys farcimen* Ehr., cells mutually accelerate their division rate by the production of a chemical agent or "autocatalyst". At the beginning of each experiment single sister cells were isolated and carried simultaneously as controls. The average per diem division rate for two animals, computed on the one animal basis, in sixteen experiments of this type was below that for one animal in the corresponding number of drops, as follows: 1 animal in 2 drops, 1.35 divisions; 2 animals in 2 drops, 1.03 divisions; 1 animal in 5 drops, 1.81 divisions; 2 animals in 5 drops, 1.54 divisions. The division rate in five drops in each instance was the more rapid, and further, there was a smaller difference between the average per diem division rate of the one animal and two animals in the five drops than in the two drops.

In brief, the data from both sets of experiments are entirely concordant and afford not only no evidence that the cells produce a substance which accelerates the division rate, but also supply further evidence that the excretion products of the cells are inimical to reproduction.

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2 Robertson, T. B., *Biochem, J.*, 1921, xv, 595; *J. Physiol.*, 1922, lvi, 404; *The Chemical Basis of Growth and Senescence*, Philadelphia, 1923, 81-105.

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ENDOMIXIS AND ENCYSTMENT IN SPATHIDIUM SPATHULA

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THREE FIGURES

Since the discovery of endomixis in Paramecium by Woodruff and Erdmann ('14), various searches have been made for similar reorganization processes in other Infusoria, but from the results it would appear that the phenomenon is far more limited in the free-living state than was at first supposed. Calkins ('21), however, has described an independent nuclear reorganization process in Uroleptus induced by separating conjugating individuals before the interchange of pronuclei. In such forms there is a significant increase in the division rate similar to that following the completion of normal conjugation (Calkins, '19). In one pair, separated accidentally before fusion was complete, the mere stimulus of contact apparently proved sufficient for starting the whole series of nuclear changes; and in explanation Calkins suggested that "the protoplasm of an individual ready to conjugate is all set, so to speak, for the succession of maturation stages leading to reorganization and rejuvenescence and that any stimulus will start the machinery."

THE EFFECT OF SEPARATING CONJUGANTS

To test the general applicability of this conclusion, attempts were made to induce a similar phenomenon in a pedigree culture of Spathidium spathula. For a study of this question the race under investigation afforded unusual advantages. There was no indication that endomixis occurs ordinarily in

this form during vegetative existence, and, as Woodruff and Spencer ('21, a, b, '24) have shown, conjugation produces a sufficient effect to make any rejuvenescence following endomixis readily detectable.

A. Method

Conjugating pairs which have just united may be easily separated by the method used by Jennings ('13) in splitting pairs of conjugating Paramecia. By repeatedly drawing them into a fine pipette and spurting them out into a large drop of medium the two loosely adhering individuals may be separated without injury. In this way a number of split pairs were obtained, each member being isolated separately on a depression slide and cultivated in the usual manner (Moore, '24). Other pairs from the same parent series were allowed to complete the process, and each normally separated exconjugant gave rise to another new series, each consisting of four lines derived from the first division products of the isolated member. In each case the four lines of the parent series from which the conjugants were obtained constituted the control.

Because of the delay in fission caused by the processes of reorganization occurring in normal exconjugants, the second or third day after separation was taken as the starting-point for a comparison of the rate of division in normal, conjugant, and split-conjugant lines. The greatest number of generations produced in each series during each five-day period was used as a fair indication of the fission rate. Since Woodruff and Spencer ('21 a, '24) found a significant increase in the number of generations attained by the majority of exconjugant lines during the first fifteen days, it was expected that any rejuvenating effect resulting from the shock of contact would be apparent in the same length of time.

B. Results

The number of generations attained by seventeen exconjugant series during each of the first three five-day periods is given in table 1, beside that of the parent series from which each is derived. A difference of three in the total number of generations on either side of that for the control is taken as essentially the same. This is necessary in order to eliminate as far as possible fluctuations arising from the method of cultivation. Even among the four lines of a series differences between the lowest and the highest number of generations may range from zero to seven. As the records show, the results are in accord with the more extensive studies already mentioned. The majority of exconjugant series attained a greater number of generations during the first fifteen days than their respective parent series. Three showed but slight variation; one died out at the end of the second period; but thirteen series produced from four to fourteen more generations. In other words, 76 per cent showed an average increase of 7.3 generations during that time—a significant increase, greater than the maximum difference found between the four lines of any series.

The history of the thirty-nine split series is somewhat different. Nine of these series died out on the same day, as the result of unfavorable environmental conditions, after only five to seven division days had passed, and another disappeared with its parent line during the third period. Several other series were lost entirely before the end of the first fifteen days, but where possible they were kept for at least that length of time. A few cultures were continued for fifteen to twenty-five days longer for further comparison.

A record of the number of generations attained by all split series and their controls during the first three periods is given in table 2. If we again consider a variation of plus or minus three to be within the limits of error, it is apparent at once that the great majority of split conjugants during the first fifteen days show no acceleration in the rate of division.

Thirty-four of the thirty-nine series produced practically the same number of generations as the parent lines, one produced six less, and three produced from four to six more. These three, 3 b, 6 a, and 6 b, were derived simultaneously from the same parent line during a state of depression. It is possible that the slight initial acceleration is more than a chance

TABLE 1

Division rate of exconjugants

SERIES	NUMBER OF GENERATIONS				VARIATION
	Period I	Period II	Period III	Total	
Control	7	12	12	31	
A a	9	14	12	35	+ 4
b	9	15	15	39	+ 8
Control	13	9	11	33	
C a	14	11	11	36	+ 3
b	11	8	—		
Control	13	9	12	34	
G a	14	13	11	38	+ 4
b	15	14	10	39	+ 5
Control	13	14	10	37	
H a	14	16	15	45	+ 8
b	15	18	16	49	+ 12
Control	10	13	7	30	
I a	13	12	11	36	+ 6
b	11	13	10	34	+ 4
Control	12	14	17	43	
K a	14	17	17	48	+ 5
Control	13	12	16	41	
L a	11	13	16	40	— 1
b	13	14	15	42	+ 1
Control	11	12	3	26	
M a	12	14	14	40	+ 14
b	12	14	13	39	+ 13
c	10	12	13	35	+ 9
Control	18	19	15	52	
N a	20	20	16	56	+ 4

TABLE 2

Division rate of separated conjugants

SERIES	NUMBER OF GENERATIONS					SERIES	NUMBER OF GENERATIONS				
	Period I	Period II	Period III	Total	Variation		Period I	Period II	Period III	Total	Variation
1 Control	13	—	—	13		12 Control	11	7	—	18	
a	12	—	—	12	—1	a	13	8	—	21	+ 3
b	11	—	—	11	—2	b	11	10	—	21	+ 3
2 Control	9	9	13	31		13 Control	10	10	11	31	
a	9	—	—	9	0	a	10	10	12	32	+ 3
b	10	9	12	31	0	b	11	9	14	34	+ 3
3 Control	6	9	13	28		14 Control	10	10	11	31	
a	9	—	—	9	+3	a	10	9	14	33	+ 2
b	10	10	14	34	+6	b	9	11	14	34	+ 3
4 Control	9	9	13	31		15 Control	7	—	—	7	
a	7	—	—	7	—2	a	9	—	—	9	+ 2
b	8	10	13	31	0	b	10	—	—	10	+ 3
5 Control	8	12	11	31		16 Control	11	10	11	32	
a	9	12	11	32	+1	a	9	—	—	9	— 2
b	8	9	11	28	—3	b	8	9	15	32	0
6 Control	6	9	13	28		17 Control	13	14	10	37	
a	10	—	—	10	+4	a	13	13	13	39	+ 2
b	11	9	13	33	+5	b	11	21	18	50	+13
8 Control	8	12	11	31		18 Control	15	19	16	50	
a	8	11	12	31	0	a	17	15	—	32	— 2
b	9	—	—	9	+1	b	15	19	17	51	+ 1
9 Control	11	9	12	32		19 Control	15	19	16	50	
a	10	11	11	32	0	a	19	13	16	48	— 2
b	11	12	10	33	+1	b	18	18	11	47	— 3
10 Control	12	11	7	30		20 Control	15	19	16	50	
a	13	8	11	32	+2	a	—	—	—	—	
b	12	9	12	33	+3	b	18	17	17	52	+ 2
11 Control	13	10	3	26		21 Control	15	19	16	50	
a	11	10	7	28	+2	a	16	19	18	53	+ 3
b	8	6	6	20	—6	b	15	20	17	52	+ 2

variation, but it is not probable that it resulted from a complete nuclear reorganization. It is, moreover, not continuous. Although series 6 a died, the four lines from 3 b and 6 b were continued for ten more days. During that time one produced exactly as many generations as the control series, while the other produced three less. Since only three cases in thirty-nine showed this increase, and since the increase itself is less than the average acceleration for 76 per cent of the exconjugants, it is not considered significant.

Series 17 b, however, shows a marked rise in the division rate and might lead to the conclusion that at least in some cases rejuvenescence does result from the stimulus of contact. There is much reason for believing, however, that the record is complicated by the occurrence of conjugation in line 2 of this series shortly after separation. During the first two days three generations were produced and four lines as usual were isolated. In the next twenty-four hours one division only took place in this line, while each of the other three closely related lines divided three times. It is just possible that the two daughter cells of an early division had mated and completed the process of nuclear interchange during that time. This possibility is strengthened by the fact that conjugation was not observed in this line until twelve days later, although conjugants continued to appear in the daily stock of the other lines. On the sixth day, when conjugation occurred on the isolation slides of all but line 2, non-conjugating individuals from this were used to replace the conjugants of the other lines. Thus the whole series for nearly two-thirds of the time included in the records consisted of animals derived from probable exconjugants.

If, one the other hand, the acceleration in 17 b had been due to an independent reorganization following contact, it might be supposed that the other member of the pair would be similarly stimulated, but no such result is noted. Only two more divisions are found in this series than in the parent line, as opposed to an increase of thirteen in the first. Furthermore, two series derived from normal exconjugants, Ha and Hb,

taken from the same parent line as 17 a and b on the same day, show an acceleration of eight and twelve, respectively, for the same length of time.

Continuation of split conjugant series is rather difficult because of the frequent occurrence of conjugation on the daily slides and the difficulty in preventing it entirely in the main lines. Series 9 a and b, however, were kept for thirty days without the possible occurrence of conjugation, and at the end of that time one had produced one generation more than the control, the other six less. It would seem that little evidence in support of the idea of rejuvenescence following contact is to be gained by a longer culture of these series.

Another circumstance which makes it extremely doubtful that any radical change goes on in most conjugants separated in the early stages of fusion is the occurrence of division without the usual delay. It is apparently possible for some normal exconjugants to divide earlier than others, but such cases are rare. In these split pairs, however, in all cases but one, each member divided from one to three times during the first twenty-four hours after separation. The only exceptions are 15 a and b, which divided on the second and fourth days. The parent series was in a very depressed condition at the time and in several lines individuals remained for two or three days without dividing and ultimately disappeared. Just what caused the separated animals to divide is questionable. It cannot be attributed to the unsuspected completion of conjugation, since conjugants appeared immediately after fission. If, as seems scarcely probable, it was due to a process of nuclear reorganization, it was not very effective, since the continued existence of series b was made possible only by allowing conjugation. Series a continued for a while, it is true, with conjugation occurring on the main isolation slides of all four lines nearly every day. No other instance like this occurred in the whole experiment, and in the absence of cytological or further physiological evidence it seems more reasonable to assign the delay in fission to the depressed condition of the parent line than to a fundamental nuclear change.

The occurrence of conjugation in split series soon after separation is another factor which seems to indicate a lack of nuclear reorganization following temporary fusion. Unless this phenomenon may be repeated in rapid succession, the facts at hand would appear to deny any such process at the time of splitting. Records for all seventeen exconjugant series show that in no case did conjugation occur within less than ten days of the time of normal separation, and in more than half of the series it was deferred for fifteen or twenty days. It therefore appears significant that of the thirty-nine series derived by splitting pairs, conjugation occurred in twenty-three before the fourth day after separation, and in thirteen others between the fifth and the tenth day. Only in three instances did the interval equal that for exconjugant series (5 a, 14 a, and 14 b), but in none of these cases was there additional evidence for believing that conjugation or endomixis had occurred. Several series, moreover, not noted in the records were discontinued almost immediately because of the presence of conjugants on all four slides as early as the second day after separation.

In the absence of any evidence of the normal occurrence of nuclear reorganization in Spathidium during the free-living state without the interchange of pronuclei, it may be asserted that the present investigation further indicates that such a process is not induced even under the most favorable circumstances. Two individuals partially fused and ready to begin the complicated series of nuclear changes involved in conjugation may be separated without detriment or benefit to either. Vegetative existence proceeds as before, with no delay and no significant change in the division rate. Conjugation is as frequent as in the parent series and occurs much sooner than in exconjugant lines. It would appear that protoplasm ready to conjugate is not necessarily stimulated by contact to complete the process, and that the " 'all or none' phenomenon" observed in Uroleptus is not of universal occurrence.

ENDOMIXIS DURING ENCYSTMENT

During encystment, however, a process of nuclear reorganization has been observed in this race of Spathidium. Regardless of their previous condition, Spathidia may be readily obtained in the encysted state by depriving them of nourishment. Vegetative forms, individuals prior to conjugation, recent exconjugants, and encysted individuals all react in the same manner when allowed to accumulate on a depression slide without the addition of Colpidia. By this simple means most of the numerous cysts studied in the present investigation were obtained. Almost any adverse condition, however, is sufficient to induce encystment, and from the occasional spontaneous appearance of cysts in day-old stock of pedigree lines it would seem that another more obscure factor is also involved.

A. Morphology of encystment

1. Dedifferentiation, encystment, and recovery. Although encystment in Spathidium has been observed by several investigators (Cienkowsky, '55; Maupas, '88; Moody, '12), no full description of the process has yet appeared. The present account of the series of stages through which an individual passes from the first indication of the preparatory phase to final emergence from the cyst is based upon the study of more than fifty mass encystments and upon a number of isolated cases. Stock individuals from pedigree cultures were set aside without the addition of food, and daily notes were made of the successive changes in the appearance of the majority of forms until the general sequence of events was apparent. Further details were obtained from individual cysts isolated at a given stage and observed at frequent intervals. Some variation was noted, especially in the rate of change, but in general the same course was followed by all.

Individuals deprived of food become clear and transparent and assume a long slender form like that shown in figure 1, A. As starvation continues, the preparatory stages of encystment

set in. The body shortens and widens somewhat and the oral region becomes less plainly marked. Gradually only a slight indication of the anterior end remains, but the contractile vacuole at the opposite extremity is clearly visible. The cell becomes more and more rounded and finally forms a perfect sphere (fig. 1, B-D). As soon as this point is reached a thin membrane appears. In one individual carefully followed through the preceding stages less than five minutes were required to complete the process—at 3:25 P.M., the cell was not quite spherical; at 3:30 it was a typical cyst (fig. 1, E).

At this stage the cyst is almost transparent, completely filled with light granular protoplasm, with a large persistent vacuole. Cysts of this kind are found on slides which twenty-four hours earlier contained normal individuals with few Colpidia. By the next day, however, a profound change has occurred. With the disappearance of the vacuole the cyst becomes contracted, its contents appear darker and more opaque, and the wall presents an irregular wrinkled appearance (fig. 1, G). An intermediate stage is occasionally found in which the protoplasm shrinks slightly away from the smooth wall, leaving a narrow space almost completely filled with granules (fig. 1, F). As the membrane contracts, however, the space is eliminated.

The opaque wrinkled condition apparently marks the most quiescent period of the whole process. Many encysted individuals degenerate at this point, but many after remaining in this state of inactivity for a shorter or longer time, usually from one to three days, begin to show signs of recovery. A small vacuole appears which gradually increases in size; the irregular contracted wall again smooths out, and the protoplasm once more transparent begins to rotate within the cyst wall (fig. 1, H-J). Signs of differentiation soon appear, and in less than an hour and a quarter a fully formed individual may be found rapidly revolving in the cyst (fig. 1, K-M). From the rounded portion containing the vacuole, in some cases, a narrow finger-like projection extends out, and as substance from the base gradually flows into this it becomes

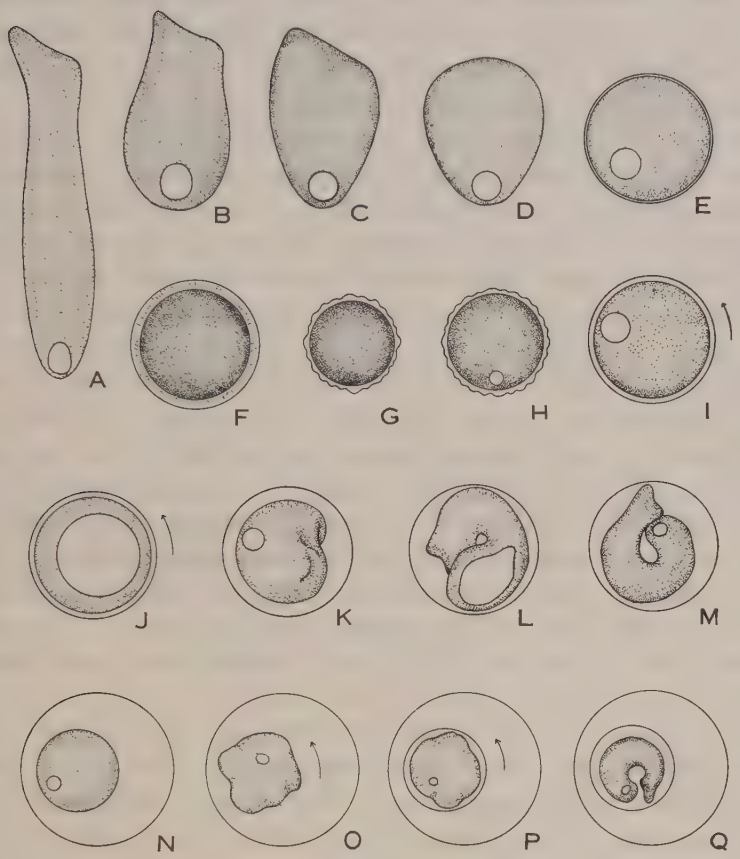

Fig. 1 Successive stages in the encystment and recovery of Spathidium spathula. × 520. A. Starved individual prior to encystment. B-D. Stages of dedifferentiation. E. Early cyst with membrane present. F. Later stage: granules extruded between cell mass and cyst wall. G. Midphase of the process: dark contracted cyst with thick, irregular wall; no vacuole present. H. Beginning of recovery; small vacuole present. I. Clear cell mass rotating within a smooth expanded membrane. J. Protoplasm circling close to cyst wall; large vacuole present. K-L. Stages in the differentiation of the encysted individual. M. Fully formed individual ready to escape. N. Reencysted individual in an early stage. O. Beginning of movement: second membrane absent. P. Movement within second cyst wall. Q. Fully differentiated individual within two membranes.

wider and more typical and finally assumes the proportions of a perfect organism, bent upon itself in conformity with the confining membrane. Occasionally division occurs within the cyst and two complete individuals are found intertwined about each other and constantly in motion.

Such fully differentiated individuals may move about in the cysts for hours without being able to escape. Unless the membrane is punctured from within or without, dedifferentiation finally sets in and reencystment occurs. The active individual gradually slows down, rounds up into a ball, and appears as a compact homogeneous mass near the center of the original distended cyst which now resembles a 'halo.' After this second encystment a second process of differentiation may occur, even resulting in excystment. A new cyst membrane is not always formed, but where it does appear the resulting individual develops within the inner wall as in figure 1, P-Q. Cysts at this stage have also been found to return to the quiescent state; but whether or not a third differentiation may occur is not known. The extrusion of granules may occur also in the cysts with halos, but by the time differentiation is complete the space is in most cases absolutely clear.

2. Process of excystment. The process of excystment involves several curious phenomena. In the first place, it is difficult to understand why some fully formed individuals are able to break through the cyst walls while others find escape impossible. Moody ('12) states that "by the addition of fresh medium it is often possible to recover Spathidium from the cysts." In the cultures which form the basis for the present study, however, no excysted individuals were observed for more than a year, although many actively revolving animals were seen during that time within the transparent membranes. Change of medium and the addition of food produced no evident effect. Drying, moreover, which Calkins ('19) found necessary for inducing the excystment of Uroleptus, proved fatal in the case of Spathidium. No further signs of activity appeared in cysts deprived of water even for a short period, and disintegration rapidly took place.

Since reversal of morphogenetic processes may be repeated several times by the active protoplasm within the cyst, it seemed scarcely plausible to attribute the failure of excystment to any fundamental defect in the cell itself which might have been caused by the internal changes accompanying encystment. It appeared more probable that the difficulty lay in the nature of the cyst wall which proved impervious to rupture from within. To test this hypothesis, it was necessary only to puncture the cyst from without and liberate the enclosed individual. For this the point of a small sharp scalpel was used, and organisms partly or fully developed were recovered from the cysts. If only a small opening was made, the active individual soon worked its way through and continued swimming rapidly through the water. Due to its coiled position in the cyst, it presented somewhat the appearance of a corkscrew on first emerging and moved in an exaggerated spiral. Many forms obtained in this manner were rounded or irregular at the posterior end, and many were long and extremely thin, typically vermiform in shape. Within a few hours, however, normal individuals of minute size were formed in the majority of cases. These were isolated in separate depression slides, and division was found to occur in the larger forms about twenty-four hours later. The descendants of one of these were kept for fifteen days, during which time forty-two generations were produced. Obviously, the failure of such forms to excyst was in no way indicative of an inherent deficiency in the protoplasm itself, since vegetative existence was resumed as soon as the barrier was removed.

The first individuals were cut from cysts on January 29, 1922. Up to that time no independent excystment had been noted at all. Daily stock slides from pedigree cultures were then put aside for the accumulation of cysts in mass, and the operations were continued. Ten days later, two normal active forms were found on a slide which had contained only cysts on the previous day. Had they emerged without aid or had their cysts been pricked accidentally the day before? To de-

termine this another slide containing cysts with fully differentiated individuals was set aside unmolested, to eliminate the possibility of external assistance. In twenty-four hours five excysted individuals were found, all of which divided within another day. After that, from time to time, occasional spontaneous excystment was found on slides containing individuals of widely diverse history. All were derived from the single pedigree series under cultivation at the time, but the actual conditions before encystment were as varied as possible. Except in one or two cases, only a few excysted individuals were obtained from a large number of cysts; but they represented cysts derived from ordinary vegetative forms, from the descendants of regenerated pieces, from recent exconjugants, and from individuals only a few generations removed from excystment.

No reason as yet can be assigned for the emergence of some individuals and the confinement of others. External conditions play no part, since cultures containing excysted forms were accorded no different treatment from many of those in which normal excystment never appeared. Moreover, in most cases only a few individuals on a slide were able to penetrate the cyst wall, while the great majority which had passed through the same external changes could not effect an escape. In one instance only was the actual process of emergence observed. The actively moving individual, having succeeded in making a small hole in the cyst wall, pushed its way 'head first' through the opening in an instant and rapidly swam away. It is interesting to note that there is no collapse of the membrane after perforation in this manner, and clear transparent spheres with a single opening may be found scattered through the culture after liberation of the enclosed organisms.

One month later, without any previous indication of change, a totally different method of excystment was observed in individuals from the same pedigree culture. In this new mode of escape, more in accordance with that described by Moody ('12), differentiation takes place chiefly outside the cyst wall.

The cyst contents begin to rotate as a granular undifferentiated mass of protoplasm, containing only a small contractile vacuole. A portion is then extruded through a small opening in the membrane and appears as a bubble of protoplasm outside the cyst, yet still attached to the larger mass within (fig. 2, A). Gradually more and more of the inner substance passes to the outside until the stage shown in figure 2, B, is reached. After this point the process continues more rapidly, and in less than than ten minutes excystment is complete. The

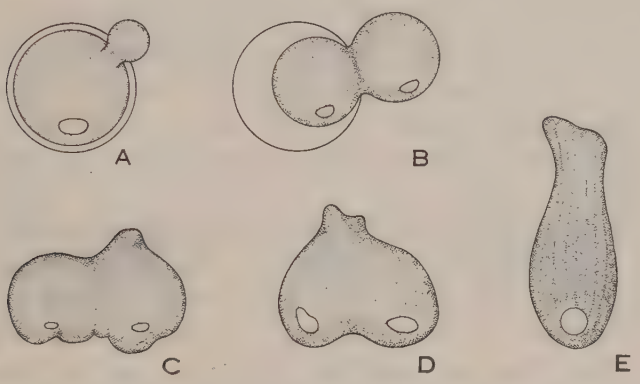

Fig. 2 Excystment of Spathidium without preceding differentiation. × 520. A. Extrusion of small lobe of protoplasm through cyst wall. B. Excystment nearly completed. C. Irregular mass five minutes later. D. Condition one-half hour after excystment. E. Fully differentiated individual one hour after excystment.

new organism at this stage may be almost fully formed or more frequently merely an amorphous mass. Such an irregular individual as that shown in figure 2, C, immediately after excystment, is quickly transformed and appears as a perfect animal within an hour (fig. 2, D, E).

B. Cytological details

In more than one hundred individual and mass preparations of cysts in various stages of the process, as well as in excysted individuals of known pedigree, the internal changes accompanying the familiar external phenomena have been carefully

studied. In several stages staining is rendered difficult by the impermeability of the cyst membrane to water. Alcoholic stains, such as crystal violet, were found to penetrate, but revealed no differentiation of the cytoplasmic and nuclear material. It was therefore necessary to rupture the wall before staining, in order to make out the internal structure of the cysts during the quiescent period. From the stage succeeding the formation of the membrane to the beginning of protoplasmic activity details were discernible only in cysts which had been cut.

Unquestionably, a process of nuclear reorganization takes place during encystment. It is possible that it does not occur in all cases, but there can be no doubt from the material at hand that at least in many instances the phenomenon of endomixis accompanies the external changes that occur within the cyst.

Long slender individuals in the starved condition invariably possess a fragmented macronucleus. As such forms become contracted the micronuclei become more distinct, staining darkly in contrast to the remainder of the cell. The bodies of macronuclear material, varying greatly in size and shape, appear at the same time less and less conspicuous. Each fragment becomes paler and more vacuolated, its outline gradually fades away, and by the time the cyst membrane appears there remains only a group of faintly staining granules loosely held together (fig. 3, A). During the stage characterized externally by a dark appearance and a thick, impermeable, wrinkled membrane, the degeneration of the macronucleus is completed (fig. 3, B). The micronuclei are quite distinct, but the only macronuclear remnants consist of chromatin granules scattered through the cytoplasm.

At the first signs of activity only micronuclei are found in the cyst (fig. 3, C), but soon after the beginning of differentiation macronuclear anlagen make their appearance. These vary in number from one to six, and strongly resemble the typical anlagen formed after conjugation (fig. 3, D, H). The micronuclei at this stage are large and contained within the

curvature of the macronuclei, as in normal exconjugants. These structures have been found both in individuals removed from the cysts and in others excysted alone They apparently remain as such only for a very short time, however, since in many cases recently excysted individuals already possess a typically fragmented nucleus (fig. 3, F). These fragments differ from those found in individuals prior to encystment,

Fig. 3 Nuclear changes in Spathidium during encystment. × 520. A. Early cyst showing degenerating macronuclear fragments and distinct micronuclei. B. Cyst in midphase with micronuclei and scattered chromatin granules. C. Beginning of protoplasmic movement: micronuclei and scattered chromatin granules present. D. Excysted individual with macronuclear anlagen and micronuclei. E. Excysted individual showing anlagen in the process of forming a definitive macronucleus. F. Excysted individual with typical fragmented macronucleus. G. Reencysted form: macronucleus fragmented. H. Exconjugant showing macronuclear anlagen and micronuclei.

consisting usually of darkly staining rod-like bodies of various lengths rather than the pale somewhat spherical granular masses already described. Intermediate stages which have been found between the first signs of change in the anlagen and the fragmented condition just mentioned further strengthen the view that nuclear reorganization has been completed in such forms, even though no evidence of the fact remains. The macronuclear bodies may be seen in the process of spinning themselves out in a kind of spiral to form long

coiled bands (fig. 3, E), and these again may be found in various stages of fragmentation, with a few long rods or many short irregular pieces.

Cysts like the one shown in figure 1, N, also possess a fragmented macronucleus much like that found in a number of individuals which have emerged from the cysts (fig. 3, G). There is thus no obvious difference in the nuclear condition of individuals which are able to escape and those which fail to penetrate the cyst wall. Furthermore, the fact that the internal structure of such cysts resembles the end stage of forms which have passed through the whole process furnishes additional evidence that these were fully differentiated forms which have reencysted.

Although it is possible that all encysted individuals do not behave in this manner and that some excysted forms possessing a fragmented nucleus have emerged with nuclear structure unchanged, yet there can be no doubt that in many cysts a complete nuclear reorganization does take place. With the macronucleus in such a distributed state that it is no longer distinguishable, it is difficult to conceive of the formation of a new macronucleus from the remains of the old. It appears far more probable that in this process, as in conjugation, where a similar disintegration of the trophonucleus occurs, the new vegetative nucleus is derived from one of the persistent micronuclei. Whether or not some of the micronuclei become absorbed in the cytoplasm while others pass through the first two maturation divisions as in the process of endomixis described in Paramecium by Woodruff and Erdmann ('14) is not known. Certain it is, however, that a new macronucleus is formed and that it passes through the same stages of development as in exconjugants.

The process of nuclear reorganization during encystment apparently bears no constant relation to the previous condition of the individual. It occurs equally in vegetative forms and in recent exconjugants, for macronuclear anlagen have been found in excysted individuals from both kinds of cysts. It is rather surprising to find such a phenomenon in cells im-

mediately after conjugation, and it is conceivable that the anlagen which appear in such excysted forms were carried through without change from the process of synkaryon formation. Even if this could happen, however, there is firm evidence that it is not the general course of events. Exconjugants in the process of encystment go through exactly the same stages as vegetative forms. The anlagen break up, become granular, and finally disappear, and a point is reached where only micronuclei are present in the cyst. There is the same need for the production of new macronuclei and the same materials with which to do it, and there can be little question that the solution of the problem is the same in both cases. There is some indication, however, that the process occurs more rapidly in vegetative forms than in recent exconjugants.

The condition of the nucleus in those individuals which excyst before differentiation is complete has not yet been determined. Micronuclei and short rod-like bodies have been observed in these cells in the process of emerging, but macronuclear anlagen have not yet been found.

C. Significance of encystment

The renewal of the whole nuclear apparatus from the idiochromatin alone has been observed in the cysts of other infusoria. Fermor ('13) found in Stylonychia during periodic encystments a process of nuclear reorganization which took the place of conjugation. This phenomenon, however, is autogamy and not endomixis, since fusion of two micronuclei preceded the formation of a new complex. In Didinium ('15) and Uroleptus ('19) Calkins has noted a degeneration of the old macronucleus and its replacement by portions of the micronuclear material, which he interprets as endomixis, but no cytological details of the process have been published.

In these three ciliates nuclear reorganization during encystment occurred only at definite periods in the life-cycle, and cysts formed merely for the purpose of protection were not

found to exhibit the same internal changes. The phenomenon of spontaneous encystment has been most carefully studied in Didinium, where Calkins followed two series through two complete cycles, each of which ended in encystment. The division rate and the rate of encystment were found to vary inversely throughout, finally reaching a point where fission ceased and 100 per cent encystment occurred. Most of the cysts, however, upon which the present study is based were obtained by depriving the organisms of food. It is therefore not known how many might have encysted without the pressure of external conditions. Since it may be questioned whether all these encysted individuals passed through the complicated series of nuclear changes which undoubtedly took place in some, it is possible that both types of encystment were represented. Any attempt to distinguish them by the interval of time between membrane formation and excystment, however, is vain, since anlagen were found in individuals which escaped in but little over a day. It is possible that the subsequent occurrence of conjugation may be used as a criterion, since conjugants were found in some immediately after excystment and in others not until after an interval of twelve days. The data on this point are as yet insufficient for correlation. Calkins ('15), however, found that in Didinium conjugation may follow spontaneous encystment on the third day after recovery.

The occurrence of endomixis in Spathidium during encystment is particularly interesting in view of the absence of such a phenomenon at any stage of its free-swimming existence. The question of rejuvenescence following asexual reorganization has yet to be studied in this form. Because of the significant effect of conjugation unquestionably exhibited by this race in pedigree cultures under laboratory conditions, it presents distinct advantages for such an investigation.

SUMMARY

1. The spontaneous occurrence of endomixis in Spathidium spathula has never been observed at any stage of its free-swimming life.

2. There is no physiological evidence that endomixis may be artificially induced in this organism by separating conjugating pairs in the early stages of fusion. Split conjugants show no such acceleration in the division rate as do ex-conjugants.

3. Cytological evidence has been obtained that endomixis occurs in Spathidium during encystment.

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REGENERATION AT VARIOUS PHASES IN THE LIFE-HISTORY OF SPATHIDIUM SPATHULA AND BLEPHARISMA UNDULANS

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FIVE FIGURES

CONTENTS

Introduction 250
Materials and methods 252
Spathidium spathula 253
A. Introduction 253
B. Regeneration in vegetative forms 255
1. Relation of regeneration to the plane of transection..... 255
2. Process and rate of regeneration 257
3. The behavior of cut pieces 261
4. The smallest pieces capable of regeneration 262
5. An attempt to produce an amicronucleate race 264
6. The division rate of regenerated fragments 268
C. Regeneration in relation to division 269
1. Process of fission 269
2. Cells cut during division 270
3. Cells cut immediately after division 271
D. Regeneration in relation to conjugation 272
1. Process of conjugation 272
2. Transections through conjugating forms 274
3. Separation of conjugants in various stages 284
4. Transections through exconjugants 285
E. Regeneration in relation to encystment 286
1. Regeneration in starved individuals 286
2. Regeneration in excysted individuals 287
Blepharisma undulans 288
A. Introduction 288
B. Regeneration in vegetative forms 289
1. Relation of regeneration to the plane of transection 290
2. Process and rate of regeneration 290
C. Regeneration in relation to division 292
1. Process of fission 292
2. Transections through dividing individuals 292
3. Transections through new cells 295

D. The rôle of the macro- and micronuclei in regeneration 299
E. Regeneration in relation to conjugation 301
1. Process of conjugation 301
2. Transections through conjugating forms 302
3. Transections through exconjugants 303
4. The effect of separating conjugants 304
General discussion 305
Summary 313
Literature cited 315

INTRODUCTION

Regeneration in the Protozoa has been the subject of much investigation and discussion, but only in the last few years have methods of pedigree culture been adopted in a study of this phenomenon. In other fields of protozoölogy, however, recent workers have clearly demonstrated the necessity of confining a given series of experiments to individuals of the same race derived from a single known ancestor in order to obtain conclusive results. Obviously, in studying the power of regeneration at various periods in the life-history of an organism, it is essential to know the condition of each experimental individual before the operation. Although this fact is gradually being recognized, the importance of the subsequent behavior of regenerated forms has been almost universally overlooked. In general, previous investigators have centered their attention upon morphological regeneration alone, and have considered the restoration of external organelles as sufficient evidence of a completion of the process. Since form regulation in the Protozoa, however, is of little value if not accompanied by the ability to continue normal existence, it would appear that a more valuable definition of regeneration is one which includes restoration of function as well as of structure. Throughout the experiments which appear in the body of this paper the criterion of regenerative capacity has been the power of a part of an organism not only to regain its typical form, but also to exist as a complete individual capable of further reproduction.

By thus differentiating between the morphological and the physiological aspects of regeneration, it has been possible,

through a comparative study of two ciliates at various stages in the life-history, to explain certain inconsistencies in the results of previous investigators, and to distinguish more clearly between the respective rôles of the macro- and the micronucleus. Although it is unnecessary at this time to review the whole subject of regeneration in the Protozoa, it may be well to give a brief summary of the present status of the problem before considering any further contributions.

Early workers[1] interested in nucleo-cytoplasmic relationships definitely established the fact that both nucleus and cytoplasm are essential for complete regeneration and the maintenance of normal functions. Gruber ('86), however, observed that restoration of form may continue in that portion of a dividing Stentor which contains the new peristome even in the absence of nuclear material. Although much of this work was carried out on dimorphic forms, nevertheless 'nucleus' was generally understood to apply to the macronucleus only. The rôle of the micronucleus still remains a matter of dispute. The process of regeneration[2] itself differs with the species, varying from a simple restitution of lost parts to complete redifferentiation from a dedifferentiated mass, and offers interesting possibilities for correlation with normal physiological processes. It would appear, moreover, that the power of regeneration is not at all times the same, but bears some definite relation to the previous condition of the organism.[3] The present experiments, undertaken with these problems in mind, include a study of the occurrence of regeneration and fission in cells of known pedigree with reference to the processes of division, conjugation, and encystment.

[1] Brandt ('77), Nussbaum ('84, '86), Gruber ('84, '86), Balbiani ('88), Verworn ('88, '92), Lillie ('96), Prowazek ('04), Popoff ('09).

[2] Holmes ('07), Balbiani ('91), Johnson ('93), Morgan ('01), Lund ('17).

[3] Calkins ('11 b) found in Uronychia a great reduction in the power of regeneration after fission, which gradually increased until at the time of the next division fragments regenerated without a micronucleus.

I take great pleasure in expressing to Prof. Lorande Loss Woodruff my sincere appreciation of his valuable suggestions and constructive criticism throughout the course of this investigation.

MATERIALS AND METHODS

Two fairly uncommon ciliates form the basis for the present study of regeneration, Spathidium spathula and Blepharisma undulans. Although presenting several distinct limitations, each form is well adapted in certain respects for experimentation in merotomy, and may be readily kept in pedigree cultures under ordinary laboratory conditions. In each case the individuals used for the experiments were descendants of a single organism isolated from a 'wild' culture in the Osborn Zoölogical Laboratory and maintained by the method of daily isolation. Except for a brief period at first when a weak hay infusion was used, the uniform culture medium throughout the experiments was a 0.025 per cent solution of Liebig's beef extract in distilled water (Woodruff and Baitsell, '11). This was prepared periodically in large quantities, poured into separate test-tubes plugged with cotton, and sterilized in the autoclave. Such a supply lasted for several months, although a fresh tube was opened each day.

Series of pedigree lines of both experimental and control animals have been kept for more than a year. Each morning, after recording the number of divisions in any line during the preceding twenty-four hours, one daughter cell was transferred by means of a capillary pipette to a clean depression slide with two drops of fresh medium and set aside in a moist chamber. In the case of Spathidium, however, another less constant factor was added. Since this organism is unable to live on bacteria alone, it was necessary to furnish it with its proper food supply, a small drop of a fairly concentrated culture of Colpidium. To prevent contamination separate pipettes were used for the isolation of Spathidia, for the 'Colpidia mixture,' and for the medium.

All operations were performed under a Bausch & Lomb binocular dissecting microscope, oculars 10, objectives F 55. Individuals at various stages in the life-history were isolated on an ordinary glass slide in a small drop of medium and transected in the desired plane. For the early experiments in cutting a finely sharpened scalpel was used. In attempting to orient certain individuals in a later series, by means of an eyelash mounted with balsam at the end of a short glass rod, it was discovered that the hair was of sufficient firmness to cleave the protoplasm even when lightly applied, and constituted a much more delicate tool than the knife for removing small portions. It was accordingly used in preference to the blade in all succeeding experiments.

Preparations for cytological study were made from time to time. As fixing fluid Schaudinn's sublimate-alcohol was used exclusively, the individuals being killed singly or in mass directly upon a glass slide smeared with a thin film of albumen, to which they readily adhered. Various stains were tried, including Delafield's hematoxylin, crystal violet, borax carmine, and picro-carmine. The latter, although requiring a longer time for destaining, has been found to give the best general results.

SPATHIDIUM SPATHULA

A. Introduction

The holotrichous ciliate, Spathidium spathula, has been va riously named and described since 1773. The most recent work on this form, however, involving a study of the structure, food reactions, and the effects of conjugation, has been done in this laboratory by Woodruff and Spencer ('21 a, b; '22 a; '24) on pedigree cultures derived from a single organism isolated from an aquarium jar November 5, 1920. The present study is based on individuals of the same race obtained from Professor Woodruff and continued in the same manner.

Structurally Spathidium offers certain advantages for the study of the effects of artificial transverse fission. Its body presents two well-defined regions differentiated anteroposteriorly, and although longitudinal cuts are almost impossible because of its slender form, transections through any plane perpendicular to the main axis are readily accomplished, and may be definitely referred to the natural divisions of the organism. The wide, obliquely truncated anterior extremity bears the mouth, and thus complete separation of the oral apparatus from the rest of the body is brought about by every transverse cut. The adjacent neck region is narrow, flattened, and much more transparent than the succeeding cylindrical portion which comprises the greater part of the organism. This is usually distended with food and fairly opaque in appearance, except at the somewhat narrowed posterior end which bears the contractile vacuole.

The macronucleus is of such character that a portion is necessarily contained in nearly every fragment resulting from merotomy. It appears in stained specimens either in the form of two long sinuous bands extending from one end of the cell to the other, or broken up into many fragments scattered throughout the body. A continuous nucleus is probably the typical vegetative condition, but it is found only occasionally in preserved forms. This may be due to the fact that individuals for staining were usually first starved for a short time to eliminate confusion arising from the presence in the cytoplasm of ingested Colpidia; and fragmentation of the nucleus invariably accompanies lack of food. Although Moody ('12) found no micronuclei in the race of Spathidium which she studied, the present organism agrees with that described by Maupas ('88) in the possession of a number of such bodies, ranging apparently from five to fourteen. Because of the chromatin fragments and food vacuoles usually contained, the exact number is, however, difficult to determine.

Spathidium is, moreover, an interesting and convenient form for the study of regeneration not only because of its structural peculiarities, but also because of its life-history.

It divides by the simple method of transverse fission, from one to five times in twenty-four hours, according to various internal and external conditions. Furthermore, in marked contrast to the form described by Moody, conjugation occurs quite readily. If it is prevented in the direct line, epidemics of conjugation may be found in day-old stock almost continuously for weeks at a time. Then non-conjugating periods of eight or ten days intervene, and the cycle of frequent conjugation is resumed. Another common phenomenon is that of encystment, which may be induced at any stage in the life-history. It is thus clear that Spathidium offers a wide range for experimentation during various phases of the life-cycle. The work on Spathidium included in this paper covers a period extending from January 4, 1921, to March 10, 1922.

B. Regeneration in vegetative forms

1. Relation of regeneration to the plane of transection. Spathidium spathula in the vegetative state possesses a very strongly developed power of regeneration. A series of operations on perfectly normal individuals under good environmental conditions demonstrated the high degree to which this restorative capacity may attain in an organism of this nature. The first experiments on merotomy were begun in January, 1921, with the idea of determining the relation of regeneration to the plane of transection. Other similar experiments in August and again a year later, in January, 1922, gave essentially the same results. Both fragments resulting from transverse cuts through any one of the three regions indicated in figure 1 have the ability to form a complete new individual capable of further division. Transections through the mid-region which produce fragments of approximately equal size yield optimum results. Both anterior and posterior portions regenerate fully, that is, both in form and reproductive ability, in 80 per cent of the experiments. In the other 20 per cent one fragment at least is lost, through various incidental causes, either at the time of cutting or before the occurrence

of fission. If, however, one part is much smaller than the other, as in operations through regions I or III, both parts recover completely in only 40 per cent of the cases; and if a very minute portion is removed from either extremity, the relative regeneration of the smaller fragment may fall as low as 12 per cent. Very rarely, however, do both parts fail to regenerate. Of 100 operations, comprising an equal number of cuts through all three regions of the body, only six gave negative results in the two fragments. The number of cases where the larger part alone maintains an independent existence is the same for transections through the anterior and through the posterior regions, regardless of the amount of regeneration necessary.

The figures just given refer only to those individuals which were able to divide after regaining their typical structure and attaining normal size. Frequently, however, a small fragment became a perfect organism as far as external appearance was concerned, but remained unable to initiate the process of binary fission. Some such instances are undoubtedly due to the mechanical difficulties of feeding, since regenerated forms are frequently much smaller than the organisms on which they prey; and in these cases the minute individual, completely formed within a few hours, disintegrated before the next day. More difficult to understand, however, are those regenerated fragments which grew to normal size and continued to live for two or four days without attempting to divide, and then disappeared. Fragments from either extremity show this behavior in 10 per cent to 20 per cent of the cases, but rarely does a regenerated portion from a mid-cut fail to divide. Only one such instance in an anterior piece is recorded after thirty-two operations. It is interesting in this connection that small irregular pieces, obtained by the rupture of an organism in contact with the surface film, frequently regenerate the normal form, but are not always capable of further division. Obviously, some factor normally present is lacking in these forms, probably due to the small size of the fragment and the region from which it was derived.

2. Process and rate of regeneration. The process of regeneration does not always follow the same course, but varies with the size and complexity of the fragment, and with the condition of the organism at the time of cutting. In a cut through the midregion variation in size between the two pieces is almost eliminated, but difference in the degree of differentiation of the new structures to be formed is clearly emphasized. The anterior portion has the simpler task and may conceivably solve its problem by retaining its highly specialized oral apparatus, and merely rounding out at the posterior end and developing here a contractile vacuole. The usual procedure, however, is not quite so direct as this. No instance has been observed where the cytostomal region remained entirely unchanged throughout the process of regeneration. After the separation of the two fragments the cut edges close immediately and each part may continue to move about for a few minutes as a typical anterior or posterior half of an individual, without undergoing any change in shape whatever. The cut end of the anterior portion then begins to round out and the tendency to assume a spherical form gradually extends to the oral region. This highly differentiated portion becomes less distinct, the neck disappears completely, and finally only an indication of the oral lips is apparent. In a number of cases the dedifferentiation proceeds even further, the fragment forming a complete sphere with all trace of the oral apparatus totally obliterated. Occasionally in a small well-fed individual each piece rounds up into a ball at the instant of cutting, but usually the process requires from fifteen to thirty minutes. In some instances a vacuole larger than the posterior contractile vacuole appears at the anterior end before all suggestion of the mouth region is lost, and in these cases it may be affirmed that the original polarity is invariably retained, as the new oral apparatus always arises at this point on disappearance of the vacuole. From a partially or wholly dedifferentiated fragment an absolutely typical, though somewhat shortened, Spathidium may develop in less than a quarter of an hour.

Elongation and the assumption of normal proportions gradually produce a perfect organism.

In the posterior piece the sharp edges at the cut surface are soon smoothed out and the fragment presents the appearance of an undifferentiated cylindrical body, rounded at both ends, which gradually becomes drawn out posteriorly. As the anterior end rounds out there is a gradual narrowing at this point, the protoplasm here becomes differentiated and in forty-five minutes a clear neck region is apparent. The process now becomes somewhat slower and an equal length of time is necessary for the formation of the mouth and the typical flared lips. Complete restoration of form in the anterior fragment requires from thirty minutes to one hour, in the posterior portion from one to two hours, or twice as long.

With diminution in size the rate of regeneration is somewhat affected. As the plane of fission gradually moves toward either extremity, the smaller fragment passes through the same stages as those already described in almost the same period of time. It is remarkable to observe a small portion from the posterior end smooth off its cut surface, elongate at once and begin to restore its oral extremity. Very small fragments from the anterior end, however irregular and jagged in appearance, round up immediately and become completely dedifferentiated before beginning the process of reorganization. Extremely small posterior fragments, however, do not always assume a spherical form. Whether because the protoplasm here is of a different texture from that at the transparent anterior end or because it is less differentiated, a small jagged portion frequently retains its shape, smooths off the irregularities and begins to move with the smaller anterior end in advance, gradually becoming organized in all parts at once. If the plane of transection is somewhat oblique, the narrowed anterior portion is retained and differentiation of the oral region begins immediately. In cases where the fragment is merely a thin slice from the posterior border, much greater in width than in length, the

original orientation is still preserved, and one anterior corner is gradually extended more than the other, the protoplasm of the other side being pulled around posteriorly to restore the normal proportions without disturbing the two main axes. In some instances a very small piece may not become typical until the next day, but all such late regenerates fail to divide. Normally the smallest pieces which regenerate are fully restored in from two to four hours.

The larger portions in these cases are usually able to complete the process in a somewhat shorter time. If the experimental animal is small, the mode of reorganization of the larger anterior fragment, resulting from a cut through zone

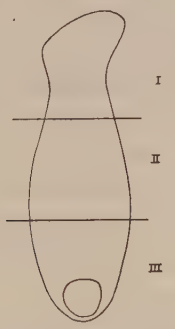

Fig. 1 Diagram of Spathidium showing the three regions through which cuts were made. × 520.

III (fig. 1), follows the same course as that after a mid-cut and requires the same amount of time. If, however, a large individual is used, there is no dedifferentiation at the anterior end. The posterior extremity soon rounds off and gradually elongates to restore the normal form. A large posterior piece, after the removal of a small portion of the cytostomal region, may be completely regenerated and able to feed within an hour. In several cases such individuals were observed to kill and engulf a Colpidium seventy minutes after the operation. Where the newly regenerated organism is not too small to compete successfully with its prey, feeding is possible as soon as the oral region regains its normal external appearance.

The function of asexual reproduction as well as of nutrition normally proceeds undisturbed in fully regenerated individuals, and the appearance of the first division depends to a great extent upon the size of the fragment. Since fission typically occurs only in individuals of average size or more, it is to be expected that the regenerated organism from a very small piece must first grow before dividing. The products of transections through the midregion which divide at all complete the process within twenty-four or thirty hours after the operation, and the same is true of fragments only slightly smaller. Very small pieces, however, from either extremity, although fully regenerated in almost the same time as the larger portions, require forty-eight or sixty hours for growth and internal reorganization before dividing. Larger fragments may divide more than once before the next day. In several instances, where only a minute portion had been removed from the anterior end, the large piece regenerated and divided in less than six hours. One of the daughter cells was appreciably larger than the other, probably indicating that the plane of fission had already been determined before the operation, and still remained effective although complete reorganization had taken place.

From these observations it is apparent that normal vegetative Spathidia possess a remarkable power of restitution. Except in forms which have recently fed, there is no loss of substance during the operation as in Paramecium,[4] where the smaller fragment invariably explodes. The wound surfaces close over at once, the outer edges being apparently pressed together by the force applied at cutting. Where dissolution does occur, it is not of the nature of a sudden explosion, but takes the form of a gradual exudation of the transparent contents of the cell through the cut surface, leaving only an outer opaque mass which soon disintegrates.

In cases where the incision is not complete the individual rounds out at once, indicating the elastic quality of the pro-

[4] Balbiani ('93), Calkins ('11 a), Peebles ('12).

toplasm and the toughness and flexibility of the pellicle. Only a slight ridge is left about the organism in the region of the cut, which may remain visible for as long as seven hours afterward. If the connection is merely a very thin thread the two fragments, separated by an appreciable space, quickly twist themselves apart as in normal fission. Such an individual, however, quieted for five or ten seconds with chloroform to prevent immediate movement, was observed to resume its normal form in less than ten minutes by the gradual distention of the delicate strand.

Although violent disruption of the cell does not follow cutting it may be induced by drying, or by pressing an individual close to the edge of a drop of the culture medium, and then immediately rescuing the products of dissolution. In this way small irregular bits may be obtained which may regenerate as quickly as the fragments from normal transection. By no means do all such pieces live, however, nor do all which regain their normal form divide; but small fragments from all parts of the body have been found which, though flattened, narrow, or extremely bizarre in appearance, are capable of perfect regeneration in two to five hours and of division a day later.

3. The behavior of cut pieces. Observations on the behavior of fragments of Spathidium derived from transections through any part of the body confirm the general results of previous investigators that pieces of ciliates move in essentially the same manner as the entire organism.

The normal reactions of Spathidium, already described by Woodruff and Spencer ('22 a), are typical for a free-swimming form which does not remain in contact with solid surfaces. It usually swims fairly rapidly, rotating on its long axis, while the extremely flexible anterior end describes a wide spiral. As a result of the tendency of the organism to swerve toward the shorter side, the more extended portion of the lips is always turned outward, whether the individual is moving forward or merely swinging about in place. A change in direction is effected by a sudden, almost

imperceptible, halt or a slight backward jerk, followed by the usual turning movement and a continuation of the forward spiral.

If an individual is cut through the midregion the two fragments begin to move immediately after separation almost as if they were still a part of the whole organism. The anterior half moves somewhat more rapidly, usually for quite a distance without reversing, pursuing the same spiral course, with the extended lip always describing the wider circle. Reversal occurs in the usual manner, and swinging movements in place are typical. Even after the piece becomes spherical it continues to revolve as it traces out its spiral path.

The posterior piece is slightly less active, and moves backward for a time, following the usual spiral, but it is impossible to determine whether it always turns toward the same side, since there is no differentiation. Circling movements in place occur also as in the normal animal.

Very small pieces from the oral end move with great rapidity, spinning about in place or swiftly advancing in a spiral course. After a time the motion slows down, but in the smallest pieces which regenerate there seems to be no period of absolute inactivity. In the larger posterior portion from such cuts an irregular waving of the anterior end is found, independent of the flared lips and their stronger cilia. Minute posterior fragments likewise react in a perfectly typical manner, and even jagged irregular pieces obtained by drying are capable of executing all the usual motions.

It was not possible in all cases to recognize a structurally defined side toward which the fragment always turned in its spiral course. It is probable, however, from the occurrence of similar reactions in pieces both large and small taken from any region, that, as Jennings and Jamieson ('02) have expressed it, "any piece of the body . . . . is organized so as to move and to react to stimuli in the same manner as does the entire animal."

4. The smallest pieces capable of regeneration. During the course of the experiments exceedingly small frag-

ments from either end of the animals were found capable of forming perfectly normal Spathidia. It seemed probable that in this organism the relative minimal organization mass of Stentor was exceeded;[5] and to ascertain this point measurements were made of the cut pieces from more than 200 individuals at various phases in the life-history. Each fragment, after isolation in a small drop on a depression slide, was transferred immediately after the operation to the stage of the microscope and its dimensions determined with the aid of an ocular micrometer. Relative volumes were calculated by means of the formula $V = \frac{4}{3} \pi abc$, where a, b, and c equal one-half of the length of each of the three perpendicular axes of the ellipsoid body. Since the length and thickness were invariably the same in the large fragments, a and c were identical, and only two measurements were necessary, but occasionally greatly flattened pieces were obtained from either end, and in these instances the three dimensions were recorded.

Table 1 includes a list of some of the smallest fragments obtained, indicating the percentage relation of the smaller portion to the total volume. As may be seen, several pieces which regenerated perfectly represent only 4 per cent of the whole, approximately the relative size of the smallest fragment of Stentor which was found capable of regaining its normal form. One extremely minute individual, which was noted at the time as the smallest whole Spathidium yet observed, represented, however, only 1.3 per cent of the organism from which it came. It was derived from the posterior extremity of an individual in the beginning phase of conjugation, and had regenerated perfectly in five hours. Unfortunately it was lost by evaporation of the small drop of medium in which it was contained while under observation. Most of the other smaller individuals which had also regenerated in the same period of time had disappeared by the next day. Of the three which represented approximately 6.6

[5] Lillie ('96) found that a nucleated portion of $^1/_{27}$ the volume of an average individual is approximately the smallest part of Stentor capable of regeneration.

per cent of the original animal, one from a conjugating pair did not regenerate until the second day, and then underwent secondary degeneration. Another lived two days as a small typical individual and then died, while another, from an organism in the vegetative condition, divided on the second day after the operation, and continued its normal existence with conjugation and encystment as usual.

Fragments cut from very small individuals immediately after fission and from extremely slender forms derived from cysts also gave rise to minute organisms. These, however, although actually as small as individual S^v do not bear the same relation to the whole. In either case extremely small regenerates do not usually remain alive very long. Undoubtedly the problem of nutrition and the general environment have some bearing upon the case, but since a small fragment may survive for several days, it seems more likely that another factor is involved. What comprises this necessary element for continued existence has not been definitely determined, but there are indications that it may be the micronucleus.

5. An attempt to produce an amicronucleate race. Micronuclei in this race of Spathidium are numerous and may be found scattered from the clear neck region to the vicinity of the contractile vacuole. Such distribution does not always occur, however, and it is possible to remove a small portion which contains no micronucleus. The question naturally arises as to whether or not such a fragment is able to regenerate and give rise to an amicronucleate race capable of continuing normal vegetative existence. Amicronucleate races of Infusoria which commonly exhibit a dimorphic nuclear condition have been definitely known since 1919,[6] although even before that time various forms had been described in which no micronucleus was observed.[7] Landis

[6] Dawson ('19) successfully bred in pedigree cultures for a period of several years a race of Oxytricha hymenostoma which revealed no micronucleus during any phase of the life-history.

[7] Thon ('05) after a careful cytological study of Didinium failed to discover a micronucleus, and Moody ('12) found none in Spathidium after running a long pedigree culture.

('20), Patten ('21), and Woodruff ('21) have since reported the discovery of other amicronucleate races, adding further evidence to the view that a single nucleus may be sufficient for ordinary vegetative existence. Conjugation is apparently impossible, however, for if cell fusion does occur it is accompanied by none of the usual nuclear changes, and the result is abortive.

TABLE 1

Relative size of smallest regenerated fragments

ANIMAL	DIMENSIONS (IN MICRONS)						VOLUME $=(4/3\pi)$ abc			RELATION OF SMALL FRAGMENT TO TOTAL VOLUME
	Anterior fragment			Posterior fragment			Anterior	Posterior	Total	
	a	b	c	a	b	c				*Per cent*
1. XV	11.28	11.28	9.4	14.1	32.9	14.1	1196	6540	7736	15.4
2. UVI	5.64	31.96	5.64	22.56	28.2	22.56	1016	14342	15358	6.6
3. DI	11.65	16.35	11.65	22.56	22.56	22.56	2219	11470	13689	16.2
4. RV	9.4	11.28	9.4	16.92	35.72	16.92	996.5	10225	11221.5	8.8
5. BVI	9.4	14.1	3.76	15.98	32.9	15.98	498.3	8402	8900.3	5.6
6. DVI	13.16	13.16	7.52	18.8	18.8	18.8	1302.7	6647	7949.7	16.3
7. RVI	9.4	9.4	5.64	14.1	28.2	14.1	498.3	5605	6103.3	8.1
8. Q	11.65	11.65	9.4	14.1	33.85	14.1	1275.5	6727	8002.5	15.9
9. IIII	9.4	22.56	7.5	28.2	28.2	28.2	1590	22420	24010	6.6
10. RIII	6.58	18.8	4.7	18.8	37.6	18.8	581.4	13292	13873.4	4.2
11. SV	14.1	35.72	14.1	2.82	9.4	3.76	7100	99.65	7199.7	1.3
12. V	18.8	37.6	18.8	6.95	11.65	6.95	13292	562.8	13854.8	4.0
13. E	9.4	9.4	9.4	14.1	65.8	14.1	830.4	13080	13910.4	5.9
14. A	9.4	16.35	9.4	25.75	47.0	25.75	1444.3	31150	32594.3	4.4
15. B	18.8	18.8	4.7	23.5	42.3	23.5	1661.5	23360	25021.5	6.6
16. C	14.1	11.65	7.5	18.8	47.0	14.1	1232	12460	13692	9.0

To determine the possibility of producing an amicronucleate race by artificial means, as Lewin ('10) claimed for Paramecium, a series of experiments were made in which only the extreme anterior tip was removed, the pieces ranging in size from one-twenty-fifth to one-sixtieth of the volume of the whole animal. Due to unfavorable environmental conditions and the unfortunate choice of long slender individuals for

cutting,[8] regeneration failed completely in 34 per cent of the sixty-eight operations. In 28 per cent regeneration, followed by fission, occurred in the larger posterior portion; in 7 per cent one piece regenerated but died without dividing; and in 31 per cent both parts regenerated the proper form without, however, continuing to reproduce in all cases. The posterior piece divided in all but three instances where both fragments formed typical individuals, but of twenty-one regenerated anterior portions only eight were able to continue normal existence.

Where fission took place four lines were isolated from the descendants of each fragment, and a careful record kept of their subsequent history. If the small regenerate possessed no micronucleus it was to be expected that its descendants would behave differently from those of the larger fragment which thus necessarily contained the full number of micronuclei characteristic of the original individual. Besides the evidence of cytological study, it was assumed from the results of previous workers than an amicronucleate condition would be revealed by the failure of such individuals to complete the process of syngamy. When conjugation was observed, exconjugants were isolated and carried along in the usual manner. Of the eight individuals that divided after regenerating, all four lines from four different fragments died in less than ten generations after the operation. Pure lines from four others, however (F, G, I, and A_3), were successfully carried on for weeks.

The history of each regenerated organism is practically the same. Individual F was cut March 7th, and the small anterior fragment isolated in the usual medium. This quickly regenerated, and on the second day had divided to form two individuals which were used to start two lines. From these, two more lines were separated the following day, and carried along until the eightieth generation. Conjugation occurred

[8] It was not known until later that such forms, in the preparatory phase of encystment, behave differently in the matter of regeneration from individuals under more favorable conditions of nutrition.

as early as the fifth generation, and appeared thereafter at varying intervals until the race was discontinued. At five different periods conjugating pairs were isolated, and in four cases they separated normally, divided the next day, and continued an active vegetative existence. The study of stained preparations, moreover, demonstrated that the process of conjugation had been complete. Maturation spindles were found in several specimens, and in one exconjugant four macronuclear anlagen and nine micronuclei were unmistakably identified. Bodies possessing all the characteristics of micronuclei had been observed in vegetative individuals, but it was considered possible that these were merely a part of the ingested food material. The discovery of distinct products of micronuclear activity in an exconjugant, however, removed all doubt of this being a typical micronucleate race.

The other three pieces behaved in essentially the same manner. Regeneration was complete and the first division took place either on the second or third day after the operation. Conjugation occurred in all three series, and exconjugants were successfully carried on for generations. In G and A_3, where the posterior fragments also were kept, no difference was noted in the behavior of the individuals derived from the two parts. Cytological study of all three races further confirmed this physiological evidence of the presence of definitive micronuclei.

Since only a small percentage of anterior fragments continued to divide, and since all of these contained micronuclei, the evidence seems to point to the essential nature of micronuclear material for further reproductive activity. Undoubtedly the failure of many regenerated fragments to remain alive for more than one day was due to their inability to obtain nourishment. In three cases, however, this difficulty was obviously overcome, for the individual continued to grow, finally attaining normal size, and yet disappeared on the third or fourth day without dividing. It is possible that these forms regenerated structurally without the presence of a micronucleus, but were unable to initiate the process of division.

6. The division rate of regenerated fragments. A comparison of the division rate of regenerated fragments with that of normal controls yields no significant results. Of three individuals from the same line one was transected through the middle of the body, one more anteriorly and one through the posterior region. The six fragments were isolated in depression slides and from the first descendants of each regenerate four lines were started. These were kept for eleven five-day periods, and the number of divisions in each of the twenty-four lines recorded daily. As a basis of comparison the total number of divisions in all four lines of each series for the period of five days was used. This tends to equalize individual variations and to eliminate fluctuations arising from differences in the time of daily isolation, without concealing any marked changes which might occur during that period. Since the first division in smaller fragments is somewhat delayed, the second day after the operation was taken as the starting-point. The curves for both fragments from each individual were then plotted with the one from the control series in order to show the relations more clearly.

No permanent effect of the operation was apparent in the resulting individuals. The records for fifty-five days were almost identical in both the experimental and the control series, and except in the beginning, periods of acceleration and of depression coincided exactly. Moreover, the greatest number of generations produced by each series during the first twenty days was practically the same as that reached by the normal animals, ranging only from one above to four below. No correlation, furthermore, was possible between this slight variation and the size of the fragment which gave rise to the series or the region of the body from which it came. In one case the small anterior fragment from a cut through the oral region gave rise to the faster series of the two, in another the posterior piece from a mid-cut produced more generations than the other.

A slight initial increase in division rate was found in five of the six experimental series but subsequent experiments

failed to confirm this accelerating effect. There is no evidence, therefore, that the stimulus of cutting with its accompanying regenerative processes produces any noticeable effect upon the division rate. The reorganization and growth in both cytoplasm and nucleus necessitated by transection is no greater than that involved in normal fission except that the amount of substance available may be greater or smaller. The result is precisely what one might expect since it is quite probable that the processes which occur in regeneration are essentially similar to those which accompany the production of two animals from a single individual in ordinary fission.

C. Regeneration in relation to division

1. Process of fission. There is a distinct variation in the division rate of Spathidium at different periods, ranging from less than one to as many as five divisions per day. The process of fission itself, from the appearance of the first external indications to complete separation, is very rapid, requiring less than an hour. Several individuals isolated before the visible beginning of division and observed at fifteen-minute intervals thereafter were each found to have produced two new animals in forty-five minutes.

Progressive changes in the cell follow in general the course outlined by Moody ('12). After growth and gradual elongation of the organism there is a slight indication of a clear zone midway between the two ends, and here a barely perceptible ridge begins to form. This becomes more marked as the rounded posterior end of the new anterior individual, and a contractile vacuole appears at one side. Behind the extended portion a constriction occurs, gradually becoming deeper, and on the side opposite the vacuole the daughter cells remain attached only by a very slender thread. As the separation between the two becomes more apparent, a new mouth is formed at the anterior margin of the posterior individual; but further differentiation of the oral region usually takes place immediately after the last connection is severed. Growth

is rapid and within half an hour the products of division present a typically adult appearance.

The nuclear condition during division is characteristic. Although vegetative forms frequently show a highly fragmented macronucleus, individuals in all stages of fission possess a coiled ribbon-like structure. In some cells this is in the form of two intertwining bands, in others four may be distinguished, and at times the various windings are so complicated as to preclude accurate description. These are continuous from one cell to the other, usually extending the whole length of each; but as Moody ('12) has already pointed out, the greater part may occasionally lie in the anterior individual. Micronuclei are visible in all stages, but the process of division in these has not been observed.

Transections were made through individuals both during and immediately after fission to determine whether the power of regeneration in Spathidium bears any definite relation to the process of division.

2. Cells cut during division. Fifteen individuals were cut transversely at various levels during the process of fission. In seven cases a small portion was removed from the anterior or posterior extremity as soon as the first external traces of division were apparent. All but one of the larger pieces divided through the original plane, and in less than two hours produced two typical individuals, one considerably smaller than the other. Except in two instances the lesser fragment had also regenerated its normal form in that time. One posterior and one anterior piece, however, of very small size had disappeared before further observations were possible.

In accordance with our definition of complete regeneration, each fragment which presented a typical external appearance was kept until division or death occurred. The two early division products of the larger piece divided either the first or second day after the operation; but only two of the five regenerated individuals from small end pieces were able to continue normal existence. One had disappeared within twenty-four hours, and two others, although remaining alive

and normal for several days, finally degenerated without dividing. One of these, killed and stained on the third day, was found to possess large bodies of macronuclear material but no certain trace of micronuclei.

Three individuals cut in a later stage of the process through the plane of fission formed two normal animals which divided two or three times before the next day. In five other cases both anterior and posterior fragments were removed from individuals in which the process was nearly completed. Separation in the original plane was soon accomplished, but in only three instances were all four fragments retained. In one individual, cut through the anterior region of both zoöids, only the two larger posterior portions regenerated and divided, the small tips disappearing within an hour. In another similar experiment one large posterior piece also failed to regenerate, but rounded out and soon disintegrated. In the third case, however, where a very small piece was excised from each end, all but the anterior fragment regenerated perfectly and divided on the second or third day. Even a small anterior piece may regenerate and divide, as attested by another experiment, although in a fifth instance degeneration instead of fission followed restoration of normal form.

3. Cells cut immediately after division. Individuals in the process of fission were isolated and carefully watched at regular intervals. As soon as division was accomplished each new cell was cut transversely through one of the three regions previously described. In no case did the operation occur later than five minutes after complete separation. Twenty-two such cuts were made, fifteen through the anterior region, four through the middle of the body, and three through the posterior extremity. Of these forty-four fragments of different sizes from various parts of the organism, thirty-three regenerated into perfect Spathidia, in from two to four hours after transection. Four of the smallest pieces were lost in the beginning and seven others had disappeared in two hours; but every other portion, large or small, formed a complete organism within a very short time. Division, furthermore,

occurred in every regenerated individual except two extremely small forms resulting from cuts through the oral region.

In Uronychia (Calkins, '11 b), where complete restoration of external structure was considered sufficient evidence of regeneration without reference to the possibility of future division, only two cases of twenty-four cut after the division period gave typical regenerated individuals from both parts. In Spathidium, however, of eighteen experiments where both anterior and posterior pieces were kept, restoration of normal form occurred in the two parts in eleven cases; and in every instance where the cut passed through the midregion both fragments regenerated and divided. In this form, at least, there is no apparent correlation between the power of regeneration and the process of fission. Both during and immediately after division certain small parts removed from either extremity round up and disappear, or regenerate without dividing, just as in the intervals between fission; but in the great majority of larger fragments regeneration at any stage proceeds undisturbed. Further discussion of the relation of this to Calkins' results on Uronychia is reserved for a later section after a consideration of the effect of similar operations on dividing Blepharisma.

D. Regeneration in relation to conjugation

1. Process of conjugation. In the race of Spathidium used throughout these experiments conjugation is of frequent occurrence. At varying intervals epidemics of conjugation may be found for weeks at a time in stock cultures left from the isolation of the previous day, and even toward the end of the period among individuals of the main line. The process is typical, two individuals becoming united at the oral ends, with the more extended anterior tip of one attached to the shorter portion of the other. At first both members of a pair retain their normal extended form, but as fusion becomes more complete all trace of oral structures is lost and the two conjugants assume a contracted appearance, somewhat resembling a

dumb-bell. In less than twenty-four hours separation is accomplished, but normally another day intervenes before fission occurs.

The nuclear changes involved present no peculiarities. The macronucleus becomes fragmented and gradually disintegrates, while the micronuclei become especially active. Because of the mass of ingested material in various phases of digestion and the numerous fragments of macronuclear substance which occupy a great portion of the cell, it is difficult to follow exactly the changes in the micronuclei. Many small spindles with distinct chromosomes may be seen, however, in

Fig. 2 Spathidium. Recently separated exconjugant, showing macronuclear anlagen and micronuclei.

the clear anterior region of individuals in the dumb-bell stage, apparently formed within the micronuclear membrane; and in recent exconjugants typical micronuclei and macronuclear anlagen are distinctly visible. In these forms, the old macronucleus has completely disappeared, and all food bodies are absorbed, so that the cytoplasm presents a smooth homogeneous appearance broken only by the presence of several more darkly staining bodies, the forerunners of the new macronucleus (fig. 2). These are typically four in number, somewhat horse-shoe shaped in side view, with a micronucleus enclosed in the curvature thus formed. Although the formation, interchange, and fusion of pronuclei were not observed there can be no doubt that such processes do occur.

2. Transections through conjugating forms. Since the nuclear content of conjugating individuals is far different from that of ordinary vegetative or dividing forms, a series of experiments was undertaken to determine the relation of regeneration to the process of conjugation. Transverse cuts were made on one or both members of conjugating pairs, through the usual three planes. Animals in various stages of the process as indicated by their external appearance were used, and the behavior of the pieces in all cases carefully recorded. These were kept in separate depression slides until regeneration and fission or degeneration and death had occurred. As degeneration occasionally followed complete restoration of normal form it was necessary to examine the fragments before the next day. Most of the records, therefore, include the condition of the cut pieces five or six hours after the operation. Here as in earlier experiments mere morphological restitution was not considered an adequate criterion of the organism's power of regeneration, and only those individuals capable of subsequent fission were held to be fully regenerated. A brief summary of the results of artificial transverse fission on conjugating forms is given in table 2.

a. Transections through one member of a pair of early conjugants. When only one member of a pair was cut the other individual, in the great majority of cases, continued to divide normally. Of fifty-eight pairs cut while apparently in the beginning phases of conjugation before the two cells had lost their typically long and slender form and contracted into the dumb-bell stage, forty-three uncut individuals after separating from the operated member resumed their normal vegetative life. In ten of the fifteen instances where the intact conjugant underwent degeneration the cut portions also failed to regenerate and divide. It is possible that here there is a normal control for the expected percentage of regeneration in conjugants, and that at least 17 per cent of the mortality is consequent upon the process of conjugation itself, and in no way dependent upon the effects of the operation. Data

upon isolated conjugants, extending over a period of more than a year, seem to confirm this suggestion. Approximately 20 per cent of exconjugants under ordinary conditions fail to divide after separation but become encysted or degenerate, and disappear within one or two days.

TABLE 2

	EARLY CONJUGANTS								LATE CONJUGANTS		
	One individual cut			Both individuals cut					One cut		Both cut
	Cut I	Cut II	Cut III	Cut I	Cut II	Cut III	Cuts I-III	Cuts I-II	Cut I	Cut III	Cut I
Total number animals	34	13	11	24	10	5	20	12	30	6	27
Regeneration and fission in all parts		3			1						
Regeneration and fission in joined parts		4	5		3	5			1	2	
Regeneration and fission in posterior parts	5			5							11
Regeneration and fission in uncut and posterior part	14	1							14		
Regeneration and fission in uncut alone	11	2	3						9	2	
Regeneration and fission in only 1 posterior part				5			1				3
Regeneration and fission in 1 anterior and 1 posterior part					2		5	4			
Regeneration and fission in 2 anterior and 1 posterior parts							2				
Regeneration and fission in 1 anterior part							6	3			1
Regeneration and fission in 1 anterior and 2 posterior parts				3				2			
No fission	4	3	3	11	4		6	3	6	2	12

More interesting is the fate of the two pieces from the operated member of the pair. If the cut was made through the extreme anterior region of an individual, leaving only a very small bit of protoplasm attached to the other conjugant, regeneration of this minute fragment rarely occurred. Of thirty-four such experiments there are only two recorded in-

stances where a very small anterior piece became a whole Spathidium, and in neither case was this restoration of form followed by fission. A larger piece, however, from a cut through the middle of the body showed greater possibilities. In nine out of thirteen cases it regained its normal form, separating from the other member in less than two hours as a small complete individual, or remaining attached for a longer time, while apparently completing the process of conjugation. Two such individuals, however, failed to divide. When the cut was made through the posterior region the larger anterior piece more frequently remained connected with the intact individual and conjugation proceeded undisturbed, the two members six hours after the operation presenting the appearance of normal conjugants in the dumb-bell stage.

The large posterior fragments from cuts through zone I regenerated and divided in the majority of cases, but in a number of other instances secondary dedifferentiation and subsequent death in two or four days followed upon either a partial or total restoration of normal form. Since only two small anterior pieces regenerated and none divided after cuts of this nature, it is of little value to compare the behavior of the two fragments from the same individual. Transections through zone III present a similar difficulty, as regeneration of the extreme posterior tip occurred only twice and was never followed by fission. Mid-cuts, however, resulting in fragments of nearly equal size, are interesting from this standpoint. With one exception, the posterior fragment regenerated and divided only when the anterior piece gave like results, although in several important instances the anterior piece behaved normally while the posterior piece failed to regenerate.

From this analysis of the records, it is apparent that regeneration in conjugating individuals, even in an early stage, differs somewhat from the process in normal vegetative forms. The fate of the fragments is variable, depending to some extent upon the size, but more closely upon some other

factor. The results are sometimes in accord with those from earlier experiments on non-conjugating individuals, and sometimes totally different. Fragments of all sizes above a certain minimum derived by artificial transverse fission from any part of the body may regenerate and divide, or they may regenerate and fail to divide as in the earlier cases. On the other hand, they may live for longer or shorter periods without regenerating at all, or they may regenerate and secondarily undergo degeneration. Before considering the new element here involved and its bearing upon the question of regeneration it may be well to review briefly the results of transections through both members of a pair, and upon conjugants in a later stage.

b. Transections through both members of a pair of early conjugants. Operations upon both individuals involve even a greater mortality. Of seventy-one pairs cut while apparently in the early phases of conjugation, 33 per cent failed to regenerate at all; and in only one instance was there complete regeneration in all four parts. In a number of cases one piece was so extremely minute that it could not have been expected to regenerate fully in a normal vegetative condition; but only seven of such pairs showed complete restoration of form and function in the other three fragments. Of forty-nine conjugatings pairs where two small pieces were removed the other two larger parts regenerated and subsequently divided in only 53 per cent of the cases. In vegetative forms, on the other hand, 95 per cent of the larger fragments continued normal existence. The small pieces, moreover, were not all below the minimal organization mass, for many remained alive and active for several days before undergoing dissolution, and fragments of the same size from non-conjugating forms frequently regenerated completely. If, furthermore, size had been the only factor, regeneration should have occurred in at least 75 per cent of the larger portions allowing for the fatality consequent upon the process of conjugation itself; and in those individuals where the fragments were all approximately of equal size the death-rate should have been extremely

low. Only one pair of individuals in ten, however, where the plane of transection passed through the midregion of each, gave results comparable with 80 per cent of those obtained on vegetative forms and it was the only case as stated above where complete regeneration of all parts occurred. In five pairs regeneration took place in one piece only from each member, and in four there was no true regeneration at all.

Unfortunately when both conjugants are used for experimentation there is no normal control as when only one individual of a pair is cut; but by correcting for the 20 per cent mortality observed in one or both members of 100 pairs of isolated conjugants it is possible to obtain comparable results.

c. Transections through one or both members of a pair of late conjugants. The experiments upon conjugants in a later stage as evidenced by the characteristic dumb-bell-like appearance of the joined individuals were less extensive because of the mechanical difficulty involved in cutting. The animals in this phase are very much smaller and nearly spherical, so that removal of any but a very small portion from either extremity is almost impossible. Transections were accordingly made only through regions I and III on one individual, and through only the extreme anterior tips of both. The results of sixty-three such operations, recorded in table 2, are essentially similar to those from cuts on earlier stages. The same combinations of parts regenerated in very nearly the same ratio, although in no case did more than two fragments form complete individuals capable of further division. Where only one cut was made regeneration failed completely in 23 per cent, the expected fatality following conjugation; but in another 36 per cent the intact individual was the only one to continue ordinary vegetative existence. Of twenty-seven pairs where both individuals were cut 44 per cent were unable to regenerate and divide, approximately the same result as that obtained with conjugants in the earlier stages.

d. Analysis of the results. The results of these experiments point conclusively to the fact that during conjugation the power of regeneration is significantly decreased. What

is the new element involved and how well does it account for the facts? Although it is conceivable, and even demonstrable, that regeneration of normal form may proceed under certain circumstances without the presence of nuclear material, it is firmly established that the continuance of normal existence is dependent upon the interaction of nucleus and cytoplasm. It seems probable, moreover, that in those forms where the nuclear material is differentiated into definitive macro- and micronuclei, the presence of a micronucleus is essential. In conjugating individuals, however, the nuclear condition undergoes a profound change. The old macronucleus in each cell breaks down and becomes absorbed in the cytoplasm; and from products of one of the micronuclei two sexual elements, the pronuclei, are formed. One remains stationary; the other migrates to the oral region, passes over to the adjoining individual and there unites with the waiting pronucleus to form a synkaryon from which both macro- and micronuclei arise. Before these have been fully differentiated, however, when only macronuclear anlagen are present in the cell, a physiological regeneration takes place, renewing the structures at the oral region which were obliterated during fusion, so that soon after separation exconjugants present the appearance of normal individuals capable of further division. The synkaryon even in its earliest stages is thus able to initiate the process of physiological regeneration.

During the series of complex nuclear changes involved in the phenomenon of conjugation, however, it would obviously be difficult to cut an individual in such a way that both fragments received equal potentialities for continued existence. It is true that both the degenerating macronucleus, scattered in small bits throughout the cytoplasm, and the products of micronuclear division may be divided between the two pieces, but it is scarcely logical to consider the resorbing macronuclear material as functioning in this connection, and in the normal course of development only one of the several micronuclei in each individual resulting from the two maturation divisions is capable of further activity. Unless it be possible

for another micronucleus to assume the functions of the one which is to form the pronuclei, it is not difficult to account for the differences between the results just recorded and those previously obtained on vegetative forms. Only one fragment can possibly contain the prospective sex nuclei before the last division, although it might be possible for the fully formed structures to be isolated in separate pieces. If the portion containing the essential elements was that in contact with the other conjugant, the interchange of nuclei could proceed undisturbed although the posterior fragment would degenerate; but if it were the isolated fragment, regeneration and fission could only occur after a kind of endomixis in both the uncut individual and the posterior piece, while the anterior would disappear.

The conditions which arise are explicable on this basis. Where the results are similar to those obtained in previous experiments on non-conjugants it is conceivable that the fragments were derived from pairs in a very early stage, in which nuclear changes had not yet started, or at least before the products of micronuclear division had begun to degenerate. Such are the cases where all, or all but the very small fragments, regenerated and divided, for in none of the earlier experiments did any of the pieces which continued to live fail to restore their normal form. In a number of cases in the present series, however, fragments of various sizes remained as such for a week or less without regaining their normal shape and reproductive activities. In some there was an unsuccessful attempt to restore the oral apparatus, and in others a complete regeneration of all the external organelles, followed immediately by degeneration and finally encystment or death. Such transient restoration of normal form might occur within six hours after the operation, or might be deferred for two days. It is quite possible that these fragments lacked the necessary apparatus for continued existence, although certain substances in the cytoplasm, probably released by the degenerating macronucleus, were sufficient for restoring normal form.

A more detailed consideration of the various groups of completely regenerated fragments with this hypothesis in mind yields certain interesting results. Regeneration of form and function occurred in all parts, as we have seen, only in four cases, and only where the plane of transection passed through the midregion of one or both members of a conjugating pair in the early stage. No such cuts were made on late conjugants, so that a comparison is impossible; but it is reasonable to suppose that the few individuals giving this result had not yet entered upon the characteristic nuclear changes accompanying conjugation. That something was happening in most of the individuals cut in this manner, however, is evidenced by the total regeneration of only 17 per cent as opposed to 80 per cent in vegetative forms.

A similar explanation is possible in the fifteen cases of early conjugants where only the uncut individual and the larger posterior fragment were able to divide, since these results are in accord with many of those from vegetative forms where only a very small anterior portion was removed. On the other hand, the fact that 40 per cent, practically the same proportion, of late conjugants cut in like manner gave complete regeneration of all but the small anterior piece makes it necessary to assume that here the interchange of nuclear material had been already effected.

Where the two parts which remained in connection with each other, after the removal of a portion from the posterior end of one or both, regenerated fully and continued to divide, it is evident that the essential mechanism for further existence was contained in the anterior regions of the cell. This occurred in seventeen early conjugants and in three late pairs. In eight cases, although the part removed was at least the size of the attached fragment, it failed to divide. Of the five pairs where only one individual was cut, the posterior fragment remained alive from two to five days without dividing, although several attempts were made to restore the oral apparatus. Of three such cuts upon both members one case is particularly interesting. Both posterior portions dedifferen-

tiated after a slight indication of regeneration at the anterior end, and one remained alive as a small incomplete fragment for four days. The other was killed and stained on the second day, and found to contain only a number of degenerating fragments of macronuclear material with no trace of micronuclei or macronuclear anlagen. In the other twelve cases only a small portion was removed, but not too small to live had it possessed the necessary structures. Such pieces remained alive two or three days without regenerating, or regained their normal form only to lose it.

Where the larger portion was removed, regeneration occurred in the isolated piece rather than in the joined parts. Of 115 cuts through zone I, on one or both members of early and late conjugants, the posterior portions alone regenerated in twenty-one cases. Where this result was obtained from multiple cuts on early conjugants it is probable that the process had not yet begun. In late conjugants, 40 per cent of which behaved in this way, it is necessary to assume that the interchange of pronuclei had been accomplished, and that the resulting synkaryon was retained in the larger fragment. The only other alternative in either case is to postulate the occurrence of a process analogous to endomixis, but since there is no other evidence that such a phenomenon ever takes place in Spathidium in the active state, there is no ground for this assumption.

It is even possible to explain on this hypothesis of the essential nature of the synkaryon the regeneration of the posterior fragment in cases where the uncut individual failed to divide. If the cut was made before the actual process of conjugation had begun, the isolated piece, in the typical vegetative condition, would be expected to react in the usual manner. The intact member, however, attached to the small anterior piece, might continue the process, but receiving no pronucleus from the other, could not form a synkaryon. Where the uncut individual failed to divide it usually remained alive several days; and in at least one instance its behavior suggests the probability of the preceding explanation. In a pair of con-

jugants in the early stage in which one individual was cut through the oral region, the small anterior piece was still attached to the other member three and one-half hours later. The next day they had separated to form one very small Spathidium and one of normal size. Unfortunately the small one was lost before it was possible for division to occur, but the larger one remained alive for six days without dividing, and finally rounded out and disappeared.

The normal individual alone continued to divide in 28 per cent of the cases where only one member was cut. Unless it be imagined that one pronucleus was isolated in one fragment and one in the other it is difficult to understand why both parts fail to regenerate in cuts through regions II and III. On the other hand, if the essential part of the nuclear apparatus were removed with the posterior piece, there would be no possibility of nuclear interchange, and unless endomixis or autogamy between two products of micronuclear division occurred, further development in the uncut individual would cease. By far the greater number of such cases, however, resulted from transections through the anterior region, and an explanation is apparent. If the pronuclei were contained in the small anterior piece, fertilization of the other member could be accomplished, but the larger posterior piece would degenerate. Although the anterior portion under this assumption would possess the necessary nuclear apparatus, it would be unable to continue normal existence because of the small amount of cytoplasm.

All parts fail to divide in 19 per cent of the conjugating pairs, both early and late, in which only one individual is cut. This may well be due to the process of conjugation itself, but where a transitory restoration of form occurs it is perhaps more logical to ascribe the absence of division to some such condition mentioned above where the pronuclei are prevented from functioning properly. In multiple cuts there can be no doubt that the operation is an important factor in the failure of regeneration, since the percentage of fatality exceeds that in normal conjugants by 16 per cent.

Without assuming the occurrence of endomixis or autogamy the other various combinations of regenerates obtained may be satisfactorily explained on the basis of one or another of the preceding conditions. Only one other case needs particular mention. An anterior piece from a cut through the oral region of a conjugant in the late stage regenerated and divided, although the other three fragments degenerated. The piece was noted at the time as being somewhat larger than usual, and it is quite probable that it completed the process of conjugation with the very small portion to which it was attached. The larger posterior fragments lacking the synkaryon were unable to remain alive.

3. Separation of conjugants in various stages. Another kind of operation was made on conjugating pairs by which the two individuals were separated at the point of fusion. Eleven long slender pairs and thirteen in the dumb-bell stage were cut apart in this manner, and their subsequent behavior recorded. Both members of six early pairs had regenerated and divided by the following day, indicating apparently that the process had not yet begun at the time of separation. In two other cases only one individual continued to live, the other becoming degenerate; and in three cases both conjugants remained abnormal and died within three days.

Separation in a late stage was more successful. Both individuals in ten experiments regenerated and divided, but in every instance but one fission was delayed until the second or third day after separation. In these cases there can be little doubt that the essential feature of the process had been completed, either before the time of cutting, or independently in each member after separation. Only one individual in the other three experiments divided, and here again there was a lapse of one day indicating an internal reorganization of some sort. The other member in one case died on the third day without regaining its typical form. Two others were apparently quite normal, but remained large and undivided until five days after the operation. At this time they were starved, killed and stained and their nuclear condition ex-

amined. A number of large irregular macronuclear fragments were observed in the cytoplasm, but no trace of micronuclei. Evidently some confusion had occurred in the process of reorganization.

Although as we have seen it is possible that the process of conjugation, interrupted at the time of cutting, was continued as a kind of autogamy or endomixis in each member separately, it is far more probable that no such phenomenon took place. The only individuals in which a postponement of fission, taken as an index of internal changes, was found to occur, were those separated in a later stage of the process; and the only pairs in which both members became degenerate were among those separated in an earlier stage. All early conjugants which continued to live had divided within twenty-four hours. It is highly probable that they were separated before the internal changes had begun, and that the stimulus of contact produced no effect. Moreover, the death of other individuals seems to indicate that separation during the course of the actual process is fatal and that each individual alone is unable to reform its nuclear apparatus.

After the completion of nuclear interchange, however, each member is a perfect whole. The similarity of these results to those in which the posterior fragments of late conjugants regenerated after removal of small anterior portions seems to confirm the truth of our hypothesis. To test more fully the possibility of the occurrence of endomixis after contact a number of further experiments were undertaken which have been described elsewhere (Moore, '24).

4. Transections through exconjugants. The last series of transections in relation to conjugation in Spathidium were made upon exconjugants immediately after separation. One individual of each pair was cut, and the other kept as a control. Of twenty-six experiments, comprising cuts through various regions of the body, all parts including the uncut member degenerated and died in seven cases (26 per cent). In 79 per cent, however, of those not unfavorably affected by the process of conjugation itself, at least one fragment was

fully restored and able to divide. Both pieces regenerated completely in only one instance, but in four other cases restoration of form took place without corresponding restoration of function. Death occurred in both cut portions in 21 per cent of the cases where the control animal continued to live.

These results indicate a lessening of the power of regeneration even after conjugation is completed. During the time of internal reorganization, however, it is obvious that the extent of nuclear material is much more limited than during ordinary vegetative phases so that it is usually restricted to one fragment. The loss of this piece in 21 per cent of the experiments is, therefore, nearly comparable to the condition in vegetative forms where fatality due to incidental causes, in one or both fragments of equal potential value, reaches 20 per cent. The greater proportion of total deaths is due to the combined effect of disturbances resulting from conjugation and from the operation.

E. Regeneration in relation to encystment[9]

1. Regeneration in starved individuals. Since Spathidia may be readily obtained in the encysted state by depriving them of nourishment, a study of regeneration before encystment becomes a study of regeneration during starvation. Of fifteen clear, transparent individuals cut before the beginning of encystment, one or both parts of six only were able to restore their normal form, and only one divided. All the other pieces failed to regenerate or encysted as soon as restitution had occurred, although food was present on the slide. Substances capable of form regulation apparently exist in starved individuals as well as in healthy forms since even a small piece of volume 4.5 per cent that of the whole resumed its normal shape. It would appear, however, that changes induced by hunger are very far-reaching and once started must continue to the end. Although a fragment may

[9] The details of the process of encystment have been given in another paper, "Endomixis and Encystment in Spathidium spathula," Jour. Exp. Zoöl., vol. 39, no. 2.

pause to regain its normal form it nevertheless immediately dedifferentiates and becomes surrounded by a cyst in order to complete the process of nuclear reorganization already begun.[10]

2. Regeneration in excysted individuals. After a short period of quiescence redifferentiation occurs and fully formed individuals may be obtained from the cysts. Transverse cuts were made upon thirty-five such animals at various intervals after excystment. Since these forms were nearly all extremely small, very minute fragments were produced so that continued existence was to a certain extent limited by the size of the regenerate and its inability to feed. The preceding condition of starvation, moreover, had reduced the reserve material to a minimum.

Of twenty-two individuals cut soon after excystment restoration of form occurred in one or both parts of all except six, but fission did not follow in all cases. The larger portion from an end cut or both pieces from a mid-cut divided in only eight instances. No regenerated individual from a very small fragment long remained alive, but pieces of equal size from the same individual behaved in the same way, degenerating or dividing together. Individuals which were allowed to feed before the operation showed some improvement over those which were kept without Colpidia until after regeneration.

After the first division the power of regeneration in excysted forms is once more normal. Of thirteen cells in the first generation after excystment, cut through various regions, 100 per cent gave complete regeneration and fission in at least one part. Although the other fragment divided in only one instance this is scarcely indicative of a decrease in regenerative ability. In several cases this piece was too small to survive, and in five individuals cut through the midregion the posterior part was lost.

It would appear, therefore, that before reorganization is complete, regenerative power in excysted individuals is less than usual, but that as vegetative existence is resumed there is a return to normal conditions.

[10] During encystment a process like endomixis occurs in Spathidium.

BLEPHARISMA UNDULANS

A. Introduction

For comparison with Spathidium many of the same experiments were repeated on the heterotrichous ciliate Blepharisma obtained from a pedigree line under cultivation by Professor Woodruff. The original individual was isolated from a laboratory aquarium November 19, 1921, and from one of its descendants a week later the present series was derived. This species is apparently B. undulans (Woodruff and Spencer, '22 b), agreeing in most particulars with Calkins' ('12) description, although differing in at least one important characteristic. It is appreciably larger than Spathidium and because of its fairly sluggish movements becomes an excellent subject for the study of regeneration. It is also more satisfactory for cytological investigation, since it feeds upon bacteria rather than other ciliates, and confusion arising from the presence of ingested bodies is eliminated. Its shape, too, makes it a convenient form for transverse cuts, being narrow and somewhat flattened anteriorly, with a wider, sack-like posterior portion (fig. 3, A). The peristome, which extends as a deep cleft along one side of the organism for at least one-half its length, is provided on the left margin with adoral membranelles and on the right with an undulating membrane. The adoral zone is continuous from the tip of the organism to the mouth region, which lies deep within the posterior portion of the peristome. The undulating membrane, a wide sail-like flap, also arises at the point where the peristome sinks into the body, but extends only half way to the anterior end. A large contractile vacuole at the posterior end periodically discharges its contents and gives place to another in process of formation.

The nuclear condition during vegetative existence is characteristic. The typical macronucleus agrees with Stein's ('67) description, consisting of two large rounded bodies, one in each half of the cell, usually connected by a slender strand. Contrary to the observation of Calkins ('12), this condition

is found in every individual stained during asexual life in the interval between division. A uninucleate condition is indicative of a change, and occurs at one stage in both fission and conjugation. Free micronuclei, moreover, are present in this race at all phases of cell life, as shown by Woodruff and Spencer ('22 b). These vary in number from five to fourteen, and are often found close to the macronucleus but never within the membrane.

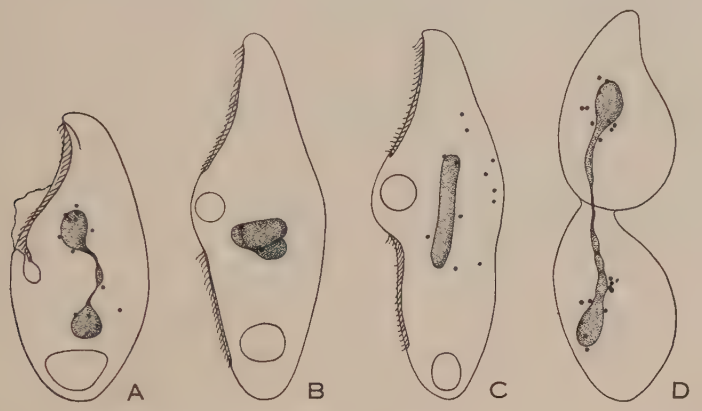

Fig. 3 Division stages in Blepharisma undulans. × 320. A, vegetative individual with typical nuclear condition. B, early division stage—macronucleus contracted (micronuclei not shown). C, division of micronuclei completed, macronucleus elongating. D, late division stage.

B. Regeneration in vegetative forms

Transections were made at various levels as in the series of experiments on Spathidium, and it was soon ascertained that Blepharisma possesses a marked power of regeneration. Although its body is highly vacuolated and its membrane is fairly delicate, no collapse normally follows the operation of cutting and both pieces swim leisurely away after separation. Its nearly constant shape gives it a rather rigid appearance but it is at the same time extremely elastic. If it is cut so that only a very thin thread remains between the two fragments, it recovers its normal form in less than five minutes and divides after the usual interval.

1. Relation of regeneration to the plane of transection. In every case one piece at least regained its normal form and continued to divide, and invariably after cuts near the mid-region regeneration of both parts was complete. After transections through the rounded posterior end the same result was obtained, unless the cut was very close to the extremity. Small anterior fragments, however, rarely regenerated and never divided; but usually rounded up into a ball, still retaining their external organelles, and becoming more and more transparent, finally disappeared completely.

The results of multiple cuts upon the same cell indicate, however, that not the small size but the lack of nuclear material is the important factor in the cytolysis of such fragments. Very small portions from the midregion which inevitably include a part of the nucleus are able to complete the process and divide, although slightly larger anterior pieces occasionally regenerate and subsequently dedifferentiate or more frequently become spherical and soon dissolve. Regeneration and fission in a small posterior piece may be explained by the fact that the nucleus usually extends closer to the posterior than to the anterior end, but here also partial restoration followed by degeneration may occur. One such fragment which was stained after it had secondarily rounded out, was found to contain no trace of nuclear material. A further consideration of this phenomenon will be taken up later.

2. Process and rate of regeneration. The process of regeneration in Blepharisma, involving the formation of more complex external organelles than the similar process in Spathidium, may be followed almost as readily in this form as in Bursaria (Lund, '17). A cut near the middle of the body leaves practically the whole oral apparatus intact in the anterior fragment, and no dedifferentiation such as that found in Spathidium occurs. There is no loss of external organelles and no tendency to assume a spherical form. Existing structures remain apparently unchanged, but soon a shifting process is evident, and gradually the typical shape is restored through a lengthening out of the posterior end. In an hour

and a half such a fragment presents a nearly normal appearance except for the relatively greater length of the peristome. Fission normally occurs within twenty-four hours.

A posterior portion from a cut of this kind possesses no trace of peristome, membranelles, or undulating membrane, but it soon acquires all these structures without first assuming a spherical form. The earliest change is indicated by an extension and narrowing at the anterior border. Then a slight groove appears, arising at the anterior tip and extending backwards. Small cilia along its course are apparently taken over to form the adoral membranelles, and in three hours an individual of typical shape appears with a somewhat shorter peristome than normal and no undulating membrane. This, however, soon arises as a small outgrowth from the margin of the peristome at its posterior extremity and grows forward. If only a small portion is removed from the anterior end so that a part of the oral apparatus is retained in the larger posterior piece, regeneration is not preceded by dedifferentiation, but the old structures are incorporated with the new. An undulating membrane in such a case is present from the start, since a piece is retained at the anterior border of the fragment. It gradually grows backward and may for a time extend the whole length of the peristome. In five large posterior pieces fission occurred within six hours of the operation resulting in two perfectly formed organisms.

Dedifferentiation, moreover, is not immediate in small anterior pieces which do not regenerate. They may remain as long as seven hours apparently unchanged from their condition at the time of cutting; and even after twenty-four hours when they have assumed a spherical form traces of the membranelles are still evident. These rounded masses, moreover, never form a membrane but become cytolyzed soon after movement ceases. Small pieces which do regenerate behave in the same manner as larger portions, restoring normal form by the use of structures already present and the shifting of materials to form new ones. In no instance was regeneration in Blepharisma found to proceed from an undifferentiated state.

C. Regeneration in relation to division

1. Process of fission. Blepharisma divides less often than Spathidium and requires a longer time for completion of the process. The external changes involved follow in general the account given by Calkins ('12), but the nuclear phenomena accompanying fission have been less clearly understood. In preparation for asexual reproduction the several parts of the typical vegetative nucleus shrink together near the center of the organism and finally become clumped into a single mass more or less irregular in outline (fig. 3, B). This is the condition of the macronucleus in every cell stained at the first external signs of fission. In such a form micronuclei are difficult to identify, but careful search reveals their presence in the form of extremely small mitotic spindles usually in the neighborhood of the larger body. An occasional micronucleus which has not yet begun to divide may also be found at this stage.

The single mass then smooths off and becomes drawn out to form the rod-like macronucleus shown in figure 3, C. Division of the micronuclei is here complete, and minute darkly staining bodies may be readily distinguished scattered through the cell in the region of the macronucleus. The short rod then rapidly elongates until it stretches almost from one extremity of the dividing organism to the other. For a time it retains the same diameter throughout its length, enlarging only at the ends, but as the constriction deepens between the daughter cells the intermediate portion gradually becomes more and more slender until only a delicate strand is left (fig. 3, D). This finally separates entirely, and by the time the last cytoplasmic connection is severed each nucleus presents the appearance characteristic of a normal resting cell.

2. Transections through dividing individuals. Organisms at various stages in the process of fission were cut transversely through both the anterior and posterior halves, and the subsequent history of the several fragments thus produced carefully recorded. Only a limited number of such experi-

ments were made in this series, but in comparison with the results already given they appear significant and may be described here separately.

Experiments 4, 6, and 8 were upon individuals in the earliest stages of division (fig. 3, B). In no. 8 there was no indication of approaching fission except a slightly elongated appearance of the cell. The plane of transection passed through the anterior region, leaving a part of the peristome near the cut margin of the larger posterior piece. The smaller piece remained practically unchanged for an hour, but at the end of two hours had regenerated a new posterior end, although the peristome still extended nearly the whole length of the organism. Dedifferentiation, however, set in the next day. The larger fragment divided in two hours, to form one perfect individual of normal size and a small anterior piece which was not fully regenerated until the next day. Both of these divided within twenty-four hours after completion of normal form.

No. 6, shown in figure 4, A, was cut in two planes, one anterior and one posterior to the developing division furrow. Both small fragments eventually underwent dedifferentiation, although the posterior piece first regained its typical shape with peristome and membranelles. The larger midpiece separated to form two individuals of unequal size both of which regenerated completely and continued to divide.

In no. 4 a very small piece was removed from each extremity of an individual in a similar early stage and the large remaining portion was then cut near the midregion. The small anterior piece rounded out at once, but the posterior piece showed an approach to normal form, with a short peristome and adoral zone. No further regeneration took place, however, and degeneration and death occurred on the sixth day after the operation. Moreover, although both larger portions formed typical individuals, only one remained normal. In two days one regenerate was able to divide, but the other gradually lost its external organelles, became spherical and disappeared on the fourth day.

Five individuals were cut at later stages in the process, two or three transections being made in each case. Experiments 3, 5, and 7 were upon cells similar to the one shown in figure 3, C, only slightly advanced over those just described. In no. 3 the second cut passed through the central contractile vacuole and resulted unfortunately in the explosion of the midpiece. The other two large fragments after five hours showed almost complete restoration of form (fig. 4, B, C). By the next day, however, their appearance was less typical,

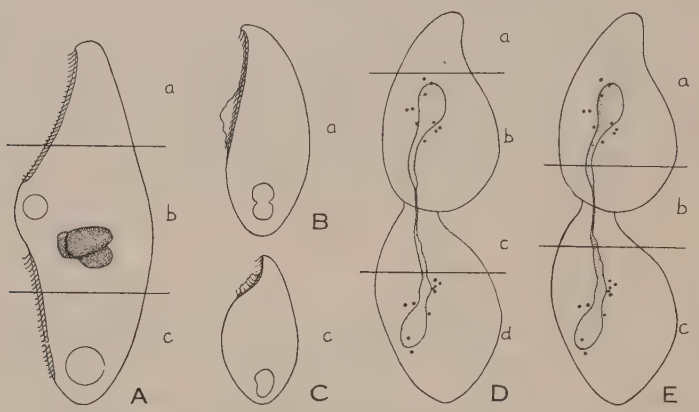

Fig. 4 Transections through dividing Blepharisma. × 320. A, exp. 6, early division stage showing multiple cuts. B, exp. 3, regenerated anterior fragment without macronuclear material—five hours after the operation. C, exp. 3, regenerated posterior fragment without macronuclear material—five hours after the operation. D, exp. 1, late division stage showing multiple cuts. E, exp. 2, late division stage showing multiple cuts.

and on the third day no trace was left of either. No. 5 was first cut in two near the normal fission plane and each incomplete daughter cell was again divided. As in experiment 4, although every piece showed signs of partial regeneration, only the larger portion from the posterior half was capable of further division. The other pieces all dedifferentiated and disappeared within four days of the operation. In experiment 7 each new zoöid was cut approximately in half but the larger midportion was allowed to divide normally. Two per-

fect individuals were formed from this part, each of which divided in two days. The anterior piece also was able to continue ordinary existence, but the posterior piece only partially restored normal form and subsequently degenerated.

Nos. 1 and 2 were individuals which had almost completed the process. A comparable stage is that given in figure 3, D. In experiment 1, a very small piece was removed from the anterior end and a somewhat larger piece from the posterior extremity (fig. 4, D). Division was accomplished through the original plane and all three larger fragments regenerated perfectly. The individual resulting from the anterior half of the posterior zoöid, however, failed to divide, although it reached normal size and remained as a perfect organism for six days after the operation. At that time, however, it appeared abnormal and subsequently degenerated. The two large posterior pieces both divided on the second day. In no. 2, where two large fragments were excised, leaving only a small portion in the constricted area (fig. 4, E), regeneration and division took place in both larger pieces within twenty-four hours, but the small midpiece disappeared in less than a day without attempting restoration of form.

3. Transections through new cells. For comparison fifteen individuals were cut at various intervals after division, ranging from the period immediately following separation to one hour later. Since no difference was noted between the results at these various times they may all be grouped together. Of nine individuals cut approximately through the midregion, regeneration and fission occurred in both parts in every instance but one. In that one case both pieces regenerated at once, but only one divided. The other became long and thin, but retained all its external organelles, and finally growing smaller and smaller disappeared on the sixth day. The manner of degeneration differed from that of other regenerated individuals after fission, but resembled a similar phenomenon found occasionally in normal cultures, to which reference will be made later. It is possible that it would have occurred independently of the operation. In five cases where only a small

piece was removed from the anterior tip regeneration and fission were limited to the large portion, but occurred in this without exception. Only one individual was cut at two levels, resulting in a very small anterior fragment which subsequently degenerated and two larger pieces both of which divided.

From the combined results of transections during and after fission and in the intervals between successive generations it would appear that complete regeneration at any time is dependent upon the same factors. Obviously cytological evidence is impossible when the subsequent history of all fragments resulting from transection is desired. One cannot stain a piece to ascertain its nuclear condition and then watch it regenerate; and after it has disappeared there is no possibility of demonstrating the absence of some essential structure. Nevertheless, from a knowledge of the normal relations of both macro- and micronuclei to the rest of the cell at various stages, it is possible to be fairly certain what portion of each is included in a given fragment after a cut through a definite region. It would appear from this that complete regeneration followed by fission can occur only in the presence of both nuclear elements.

Very small portions from either extremity of individuals during any phase of vegetative existence fail to divide. It is evident from a glance at the normal nuclear condition that such pieces are beyond the reach of the macronucleus, although a stray micronucleus may extend that far. Somewhat larger posterior pieces frequently regenerate completely, although anterior fragments of the same size rarely do so. As we have stated before, however, the macronucleus usually extends more nearly to the posterior than to the anterior extremity. All other fragments from transverse cuts on vegetative individuals, necessarily containing a portion of both nuclear constituents, regenerate and divide in 100 per cent of the cases. Even a small portion from the midregion, of the same size as end pieces which fail to regenerate, is capable of continued existence. All individuals possessing a characteristic

vegetative nucleus, regardless of their relation to a preceding or succeeding period of fission, react in the same manner after merotomy.

In dividing cells, moreover, the same dependency upon both nuclear elements holds true. When the trophonucleus is in the compact form shown in figure 4, A, large pieces from either end are unable to complete the process of regeneration. If the macronuclear mass is isolated in one small portion as in experiments 4 and 5 only that portion continues to live normally. If, however, fission is allowed to proceed through the original plane as in nos. 6 and 8, two complete individuals result which are capable of further division. At a later stage, where the nucleus has elongated, other pieces are found to regenerate fully. It is important in this connection to note, however, that in a dividing cell which has nearly completed the process, pieces from the midregion do not divide, although complete restoration of form may occur (experiments 1 and 2). In experiment 1 (fig. 4, D) fragment *c* regenerated and remained normal for five days before signs of degeneration appeared. Pieces without macronuclear material, however, dedifferentiate immediately after attainment of normal form, and those which behave like occasional individuals in the control culture shrink in volume and become transparent without losing their external organelles. This individual, however, did not become abnormal until the sixth day, and in the meanwhile had reached normal size, and presented an unusually opaque appearance. It behaved in general like regenerated fragments of Spathidium in which it was believed a micronucleus was wanting. From the normal distribution of micronuclei in a dividing cell at this stage it is evident that a similar explanation is possible in this case also.

Complete regeneration in Blepharisma is no more frequent in dividing forms than in individuals immediately after fission. Nevertheless, it is clear from the examples already quoted that restoration of normal form does occur, during the process of fission, in fragments which obviously lack the necessary apparatus for asexual reproduction. Such regen-

erated individuals, however, partially or fully restored within a short time, soon dedifferentiate, and finally become spherical and disappear. It is true, as Calkins ('11 b) found for Uronychia, that the power of form regulation is greater in dividing individuals than at any other time, but it is a mistake to suppose that all such regenerated individuals are of equal potential value. The same proportion continues to divide as in vegetative cells; the others degenerate. Calkins, however, did not keep his cells until fission took place, so that his work furnishes no evidence that fragments without a micronucleus are capable of dividing. In fact, he notes in one case that the amicronucleate portion became 'smaller and emaciated' and finally died after ninety hours. In another case where a dividing cell in the midstage was cut longitudinally the portion containing the micronucleus completed division, but the other failed to divide, although it did restore its normal form. In no instance did he find regeneration in the absence of a portion of the macronucleus.

Apparently the only previous record of the regeneration of a fragment in the absence of macronuclear material is that given by Gruber ('86) for an enucleated portion of Stentor. He found that a piece without a macronucleus from a dividing individual, in which the beginnings of a new peristome had already appeared, was capable of developing into a perfect organism, but he made no reference to its later history. Furthermore, he knew nothing of the micronuclear content of his regenerated fragments, but stated that he had been able to discover no influence which the micronucleus might exert over the process.

Obviously, unless regenerated fragments are retained until division or degeneration takes place, it is impossible to know with certainty just what factors are necessary for continued existence. It is then necessary to supplement the facts thus obtained with cytological evidence. For this purpose a new series of experiments was undertaken.

D. The rôle of the macro- and micronuclei in regeneration

One hundred and six individuals in vegetative and dividing stages were cut in such a way as to include most if not all of the macronucleus in one fragment. This was then killed and stained while the other was isolated and carefully followed with sketches and notes of succeeding changes. Since the condition of the macronucleus had been carefully determined at various phases of asexual life, and since in each stage it was found to preserve a fairly constant appearance, it was not difficult to ascertain from the stained specimen whether or not a portion had been included in the living fragment. Because of the number of micronuclear bodies in Blepharisma it is almost impossible to obtain pieces without at least one micronucleus, although there is an indication that midpieces from an organism in the late stages of fission are lacking in this structure (fig. 4, D, E).

What is the subsequent history of fragments possessing only a micronucleus? There seems to have been no previous work on dimorphic Protozoa with this question in mind. Is it the presence of a micronucleus which causes partial restoration in fragments of dividing forms with no macronuclear material? Or is it possible for a micronucleus to give rise to a macronucleus, as in the process of endomixis?

A brief summary of results is given in table 3. Twenty-nine pieces regenerated completely and divided normally; thirty regenerated but failed to divide; and forty-seven showed no signs of restoration whatever. In every case where functional regeneration was complete the stained portion indicated that a piece of the macronucleus had been included in the living fragment. Moreover, stained specimens of these regenerated individuals immediately after fission exhibited a perfectly normal nuclear condition like that in figure 3, A. Only five fragments which presumably contained macronuclear material failed to divide. Two of these disappeared soon after the operation, but the other three regenerated as usual and remained alive for four days, becoming gradually more and

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more slender and transparent, but still retaining all their external organelles. At this time they were killed and stained, and found to possess both macro- and micronuclei. Since, however, this wasting-away phenomenon occurs in ordinary cultures and may be followed by recovery, it is apparently wholly independent of the operation.

Regeneration followed by immediate dedifferentiation occurred in two anterior and twenty-four posterior pieces, all of which were in the early phases of division. The macronucleus in this stage is in a condensed mass, and it was clearly seen in each case that the whole of it was contained in the

TABLE 3

BEHAVIOR OF FRAGMENT	ANTERIOR PIECES				POSTERIOR PIECES				TOTAL
	Macronucleus absent		Macronucleus present		Macronucleus absent		Macronucleus present		
	Veg.	Div.	Veg.	Div.	Veg.	Div.	Veg.	Div.	
Regeneration and division			9	1			17	2	29
Regeneration and degeneration ..		2	3			24	1		30
No regeneration..	28	6	1		7	5			47

prepared specimen. Moreover, after it was thoroughly demonstrated that the dedifferentiated regenerates ultimately disappeared without dividing, a number of such degenerate forms were stained, and in no instance was macronuclear material found. The physiological evidence of the preceding experiments is thus confirmed by cytological study, and there can be no further question that in the absence of a macronucleus restoration of external organelles may proceed if the first steps toward the formation of a new individual have already begun. From a posterior fragment possessing only a part of the developing membranelles an organism of perfect form may arise with peristome, adoral zone and undulating mem-

brane. The mouth never appears, however, and degenerative changes immediately set in.

The micronuclear content of such fragments is of particular interest. Of one anterior and ten posterior pieces which had experienced exactly the same degree of differentiation and dedifferentiation, six were found to possess micronuclei while the others showed no nuclear structure at all, being either completely homogeneous or highly vacuolated. The micronucleus apparently plays no rôle in form regulation, although it is necessary for reproduction. It is evident, too, that it is unable under those conditions to give rise to a new macronucleus although a priori such a possibility might not be inconceivable. From one to six micronuclei have been distinctly observed in completely spherical fragments, but they showed no signs of activity. If endomixis does occur in this form it would appear that it may not be artificially induced by cutting.

E. Regeneration in relation to conjugation

1. Process of conjugation. In this race of Blepharisma the external features of the process agree with the description given by Calkins ('12), but contrary to his observations as shown by Woodruff and Spencer ('22 b), there is no fatality after conjugation. Separated individuals divide after two or four days in approximately 100 per cent of the cases. The nuclear condition of individuals recently united differs in no respect from that of typical vegetative forms (fig. 5, A), but as fusion becomes more complete the several portions of the macronucleus gradually shrink together (fig. 5, B), becoming more and more condensed until they finally form a massive oval or spherical body near the center of the cell. This may be readily distinguished from the irregular clumped nucleus which is found in the early stages of fission by its smooth contour and the frequent presence of a cleft through the center. Unlike Spathidium the macronucleus does not undergo degeneration by breaking up into numerous bodies, but becomes

more and more concentrated. Its material is probably given off to the cytoplasm in the form of minute granules, since a haze of darkly staining substance is frequently found in the region of the old macronuclear mass.

During these changes micronuclei are not visible as definite bodies, but there are indications of extremely small spindles in the anterior half of the cell. Although the maturation stages have not been worked out, satisfactory evidence of the completion of conjugation is furnished by the presence in normal exconjugants of macronuclear anlagen and distinct micronuclei. The new macronuclear elements are highly characteristic, ranging from two to six in number, and present the appearance of large pale spheres, each usually enclosing a small darkly staining body (fig. 5, D). The micronuclei, however, are not confined to this position within the anlagen, but may be found in any region of the cell. The transformation of these structures into the typical nucleus has not been observed. Thirty-six hours after separation each cell appears but little changed from its condition twelve hours after completion of the process, except that by this time the last traces of the old macronucleus have completely disappeared. With the first division, however, the normal vegetative state is established.

To test the regenerative power of Blepharisma with reference to conjugation, transections were made through conjugating pairs, and through exconjugants at various periods after separation.

2. Transections through conjugating forms. A very limited number of experiments were made upon individuals in the process of conjugation, and in every instance the results corresponded with those from vegetative forms. No small pieces from either extremity remained alive more than one day, but both larger fragments from five anterior and posterior cuts upon the two members of a pair were able to continue normal existence. Fission occurred from two to five days after cutting, according to the size of the fragment and possibly the stage at which transection occurred. In

the absence of cytological evidence, it may be justifiable to assume that where one anterior and one posterior or both posterior pieces regenerated completely the process of conjugation was either just beginning or just ending. It is possible, however, that such regeneration is due to a nuclear reorganization proceeding in each fragment without synkaryon formation.

3. Transections through exconjugants. Twenty pairs of conjugants were isolated on separate slides until completion of the process. One member was then stained to show the

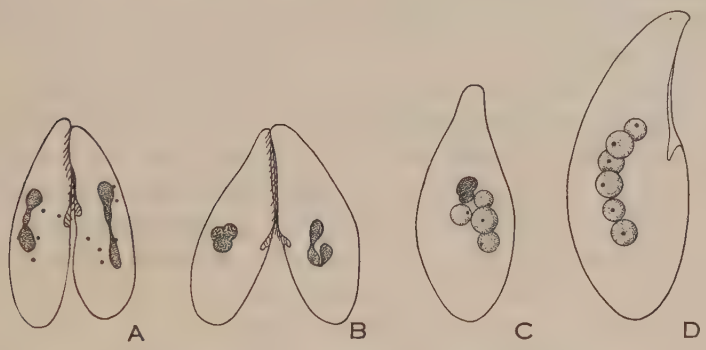

Fig. 5 Nuclear changes during conjugation in Blepharisma undulans. × 320. A, early fusion stage—nuclear condition typically vegetative. B, later stage—macronucleus contracted (micronuclei not shown). C, exconjugant showing degenerating macronuclear mass and four anlagen of the new macronucleus with micronuclei enclosed. D, exconjugant showing six macronuclear anlagen, each with a micronucleus. All traces of the degenerate macronucleus have disappeared.

nuclear condition, and the other cut to determine the regenerative power of recent exconjugants. Every stained specimen was found to contain from two to six typical anlagen, gathered near the center or extending in a longitudinal row through the cell. In the majority of experiments the results coincided with those from vegetative forms, indicating that anlagen are capable of bringing about normal regeneration. Of fourteen individuals cut through either extremity, the larger part regenerated and divided within four days in eleven cases, and in one the smaller piece also successfully

regenerated. In the other cases where the larger piece, apparently fully restored, disappeared after two days without previous signs of degeneration it is probable that external conditions were at fault. Where the cut passed through the midregion both fragments continued to live in four cases out of six. Regeneration occurred in the other fragments but dedifferentiation followed after several days. One degenerated individual which had become perfectly spherical was stained on the eighth day, and found to contain ten micronuclei and an abnormal macronucleus. The anlagen had apparently developed into a typical nucleus, since there were two large masses connected by a short strand, but secondary degeneration was suggested by their vacuolated appearance.

After the first division the results agree exactly with those upon vegetative individuals. Twenty experiments were made upon exconjugants and their descendants from three to six days after separation. On the third day, immediately after the first division, a slight fatality was found, but later all except very small pieces regenerated perfectly and divided in twenty-four to forty-eight hours.

4. The effect of separating conjugants. Five conjugating pairs were transected through the plane of fusion, and all ten of the resulting individuals remained alive and divided on the fourth day after separation. The delay in fission would seem to indicate that nuclear reorganization of some kind had occurred. Had it been preceded by synkaryon formation or was it a process like endomixis?

An attempt was made to answer this by obtaining conjugants in an early stage and forcing them apart by squirting from a pipette. In this manner twenty-one pairs were split. Both members were kept in a number of instances, but in several cases one individual was stained, either immediately after separation or at some later time before division occurred. In eight individuals which divided within twenty-four or forty-eight hours, the nucleus at the time of separation, as indicated by the stained specimens, presented a typical vegetative appearance or revealed but slight evidence of the beginning of conjugation.

Where fission was delayed for three or five days, it appears that in some pairs the essential act of conjugation had been completed before the splitting. In two individuals stained immediately after separation the old macronucleus was concentrated near the center of the cell, definite micronuclei were present, and small clear spheres, apparently the early stages in the formation of anlagen, were visible. Another presented essentially the same appearance twelve hours later, and three others, stained twenty-four or forty-eight hours after separation, showed four distinct anlagen with micronuclei enclosed. Unfortunately the condition of these at the time of splitting is unknown. It is suggestive, however, that in one case division was deferred for four days although the nuclear state at the time of separation was typically vegetative.

All attempts to settle the question on physiological grounds have proved useless, since as yet no evidence has been obtained of an increased fission rate after the completion of conjugation. This fact in itself suggests the possibility that endomixis in this species is a normal periodic phenomenon.

GENERAL DISCUSSION

The high regenerative power of both Spathidium and Blepharisma in the vegetative state under normal conditions makes it particularly simple to detect a difference at other periods in the life-history. The results are not complicated by difficulties arising from the operation itself. With but few exceptions all fragments produced by transection survive the process, and swim actively about, although the smaller pieces may undergo dissolution within a short time, and others may regenerate in form but not in function. Because of differences in the macronuclear condition of these two organisms, moreover, a comparative study reveals interrelationships of nucleus and cytoplasm more clearly. A very definite limitation, however, to a conclusive study of the essential nature of idiochromatin arises from the presence in both forms of numerous micronuclei scattered throughout the body.

The process of regeneration varies to a certain extent in the two species, but no correlation is apparent between the degree of specialization of external organelles and the method of restitution. Fragments from undifferentiated portions of the body become transformed into whole individuals by a gradual reshaping and differentiation de novo, as in other Protozoa capable of regeneration; but pieces from more complex regions show a somewhat different behavior. In neither form does dedifferentiation of remaining structures proceed to the point described for Stentor and Bursaria, although Spathidium closely approaches that state. Anterior fragments of this ciliate, possessing the oral apparatus, become rounded out and lose all visible trace of differentiation, but from the rapidity and abruptness with which a complete organism arises from the spherical mass it would seem that the dedifferentiation is merely superficial. Blepharisma, on the other hand, which has developed a greater variety of peristomal organelles, retains all its external structures, and like Loxophyllum (Holmes, '07) is able to derive a new peristome from a portion of the old.

It is possible that the spontaneous reversal of morphogenetic processes, which has been noted in both Sentor (Balbiani, '91; Johnson, '93) and Bursaria (Lund, '17) under normal conditions, may account for the occurrence of a similar phenomenon after injury, since the replacement of organelles already present and functional has never been observed in either Spathidium or Blepharisma. Preparatory to encystment, however, Spathidium undergoes complete dedifferentiation, but a like process in Blepharisma apparently does not take place.[11] No cysts have ever been seen in the cultures under observation, and fragments which round out and disappear retain traces of membranelles almost to the end. It is evident that the degree of dedifferentiation preceding regeneration closely parallels the degree of dedifferentiation accompanying normal processes, and it is possible that more

[11] Further studies on Blepharisma, however, have revealed the occurrence of encystment in this species.

than an incidental correlation exists between the two series of phenomena. It would seem that reversibility of organization is more limited in certain Protozoa than in others, but complexity of structure is not the controlling factor.

Although the process of regeneration is more direct in Blepharisma than in Spathidium the regenerated individuals from both organisms possess for a time characteristics of the part of the body from which they were derived. Moreover, regardless of the amount of dedifferentiation, cut pieces continue to react in the same manner as the entire organism. The original polarity is retained, and in certain cases movements aid materially in restoring an irregular fragment to the normal shape of the organism.

That another more fundamental cause, however, is effective in regeneration is evidenced by the fact that some fragments regenerate, while others with exactly the same reactions either soon become spherical or remain alive for days without regaining their lost organelles. Small pieces from either extremity of Blepharisma fail to regenerate, while portions of the same size from the midregion are fully restored, and in Spathidium even certain very small end fragments are able to form perfect whole individuals. During division and conjugation, however, these results may be reversed. Pieces of Blepharisma which would ordinarily degenerate may temporarily regain their normal form and subsequently dedifferentiate if taken from dividing organisms, and fragments of Spathidium which develop normally in the vegetative and dividing states may remain alive without regeneration if obtained from individuals in the process of conjugation.

Complete regeneration followed by fission is dependent entirely upon the status of the nuclear elements. With changes in the extent and condition of the macronucleus and the distribution of the micronuclei, differences arise in the behavior of pieces derived from transections at various levels. To a certain degree it is true, as we shall point out, that restoration of form is influenced by the physiological condition of the organism, but since that condition is apparently expressed in

terms of nuclear activity the same cause is operative. Although it was definitely established some years ago that the interaction of nucleus and cytoplasm is the effective agent in regeneration, the respective rôles of the two kinds of chromatin material in Infusoria have remained uncertain up to the present time. The results of these experiments, however, throw some light upon the problem.

The macronucleus in dividing Spathidium extends throughout the organism just as in the normal trophic state, and the micronuclei are similarly scattered throughout the cell. Fragments obtained both during the process of division and immediately after fission have a similar nuclear content and give rise to the same proportion of regenerates capable of further reproduction. In dividing Blepharisma, however, a difference is apparent. Although practically the same percentage of pieces continue normal existence as after division, a large number undergo partial if not complete restoration of form, followed immediately by dedifferentiation. Portions from either end, which ordinarily round out and disintegrate because of the absence of macronuclear material, may show an approach to normal shape; and larger enucleate fragments, obtained during the stage when the macronucleus is concentrated into a single mass near the center of the cell, may form typical individuals. The mouth never breaks through, however, feeding is impossible, and gradual dedifferentiation finally results in a spherical body.

In dividing individuals form-regulation is possible in the absence of definite nuclear material of either sort. As Gruber ('86) reported for Stentor, an enucleate fragment which bears traces of a new peristome is capable of finishing the structure and of producing a new organism of characteristic external appearance. This transient morphological regeneration in no way opposes the conception of the macronucleus as the form-maintaining constituent of the cell, but rather furnishes additional weight to this view. A process initiated under the influence of the nucleus is carried to completion in its absence, and then undergoes collapse. Although the fragment may

remain alive for several days, the condition of perfect external restoration lasts only a few hours. The complete cycle of differentiation and partial dedifferentiation is accomplished in less than one day. The fact that in the absence of chromatin material normal form is lost even before trophic phenomena cease points to the conclusion that the nucleus is especially active in preserving normal proportions. It would appear that substances in the cytoplasm capable of carrying out the progressive changes accompanying fission had been reduced to a minimum by a similar demand following transection, and required replenishing by nuclear activity. As Calkins ('11 b) has observed in the case of Uronychia, these facts "lend support to de Vries' and Hertwig's view that the nucleus gives off to the cytoplasm during the vegetative stages certain formative substances perhaps of the nature of enzymes, which are exhausted with the regenerative processes accompanying division."

Experiments on dividing Blepharisma furthermore definitely establish the fact that in organisms possessing a dimorphic nucleus it is the macronucleus which is essential for the maintenance of form. The regeneration of fragments containing only macronuclear material, obtained by cuts upon dividing Uronychia, scarcely proves this point since in Blepharisma similar regeneration may occur in fragments lacking chromatin entirely or possessing only micronuclear substance. The presence of micronuclei, however, even to the number of six, exercises no influence upon the regenerated fragment in the absence of a macronucleus. Dedifferentiation sets in as soon as normal form is attained, and results in the same spherical mass as in pieces without nuclear material of any kind.

In conjugating forms, moreover, as illustrated especially by the extensive experiments on Spathidium, temporary regeneration of structure is frequently found. In cases where one fragment contains the synkaryon or its products the other makes an attempt at restitution, but immediately degenerates after regaining its characteristic form. It is probable that

substances liberated during the disintegration of the macronucleus are sufficient for restoration of form but not for its preservation. In exconjugants, also, the same thing is true, and fragments loaded with material from the resorbing macronucleus may partially regenerate even in the absence of macronuclear anlagen.

It is possible that the activity of the new synkaryon, by releasing certain substances in the cell for the completion of physiological regeneration after the separation of the conjugants, is an additional factor in the temporary form regulation of amacronucleate fragments. This interpretation is strengthened by the fact that cells in the starved condition after fragmentation and early resorption of the macronucleus rarely regenerate, but more frequently become encysted. Reserve material in the cytoplasm has evidently become exhausted, and fragments continue the process of dedifferentiation already begun. As suggested previously, the physiological condition of the cell ultimately resolves itself into a question of nuclear activity, and the study of regeneration as indicative of the physiological state of a unicellular organism coincides with the study of regeneration in relation to the condition of the nucleus.

If the trophonucleus is the form-maintaining constituent of the cell, what is the rôle of the micronucleus in regeneration? No absolutely conclusive evidence has yet been brought forth, but it would appear from various results that the micronucleus is essential for further division and the continued life of the organism.[11] Every stained regenerate of Spathidium or Blepharisma which had completed division was found to possess micronuclei. In forms which contain so many micronucleate bodies it is obviously difficult to obtain fragments without at least one, but from the anterior region of Spa-

[11] Since the preparation of this paper for publication, additional evidence has appeared in support of the view that the micronucleus is essential for division. Young ('22), working on several species of Uronychia which possess only one or two micronuclei, has clearly demonstrated the fact that although amicronucleate fragments may regain their normal form they are unable to continue normal existence.

thidium it is possible to remove a piece lacking this structure. The only portions which continued to divide possessed micronuclei and successfully completed the process of conjugation. A few others regenerated the typical form and remained alive for several days, growing in size and apparently exercising all normal trophic functions, but appeared unable to initiate the process of binary fission, and after a time disappeared. The presence of a macronucleus in such pieces is obviously assured by their behavior, the absence of a micronucleus may be ascertained only by cytological study.

It is not contended that all regenerated fragments which remain normal but fail to divide lack a micronucleus. External causes may play a part and internal disturbances following conjugation may possibly prevent division. In Blepharisma, moreover, individuals in normal lines, in which a micronucleus is demonstrable, occasionally prove unable to undergo division, but become smaller and more transparent and after a longer or shorter period in this state either recover or disappear. The meaning of this phenomenon is not known, although it is possible that nuclear reorganization occurs at this time. Some regenerated individuals present this condition; but others, notably from regions in a dividing cell which normally contain no micronuclei (fig. 4, D, E), behave in a totally different manner. There is no immediate degeneration like that which occurs in the absence of a macronucleus, and there is no apparent shrinkage accompanied by unusual transparency such as in the process just described. Instead, the cell remains typical for several days and presents a somewhat opaque appearance. Then finally death occurs with or without signs of preceding dedifferentiation. Since in both Spathidium and Blepharisma it is possible to obtain fragments in which micronuclei are apparently lacking, and in which the same series of changes takes place, it would seem that it is the presence of micronuclear material which initiates the process of fission.

Only one case has been recorded (Lewin, '10) in which it was believed an amicronucleate race had been created by

artificial means, and that appeared only in a brief preliminary paper. Since transections through the nucleus of Paramecium, except in dividing forms, usually result in loss of both parts, it would appear rather difficult to remove a portion containing only macronuclear material. The regeneration of portions of Uronychia (Calkins, '11 b) in the absence of a micronucleus is easily understood. Had fission occurred in such regenerates there would be no further case for the essential nature of this organelle, but in no instance was division reported. The possibility of producing an amicronucleate race by means of transection yet remains to be proved.

In the meanwhile it appears that evidence is accumulating in support of the view that idiochromatin is indispensable for normal vegetative existence, and yet this raises certain other difficulties. In amicronucleate races found in nature it has been clearly demonstrated that processes involving the activity of a definitive micronucleus are impossible; but asexual reproduction continues undisturbed. Obviously, one of two alternatives must be true. Either micronuclear substance is not essential for division, or some difference exists between amicronucleate races spontaneously arising and those artificially produced. If it be proved beyond question that regenerated individuals are absolutely unable to divide in the absence of a micronucleus, then it may be possible to account for the viability of normal amicronucleate races by assuming an irregularity in nuclear reorganization following conjugation or endomixis. As Woodruff ('21) has suggested, it is conceivable that by suppression of the 'differential micronuclear division' at such a time, all the micronuclei may be transformed into macronuclei with the potentialities of both tropho- and idiochromatin.

Gruber ('93) found that in conjugating Stentors regeneration of fragments occurred only after a new macronucleus had developed. Experiments with Spathidium, however, indicate that after synkaryon formation pieces containing the united pronuclei are capable of continued existence, although tran-

sections prior to nuclear interchange result in the loss of all parts. It is evident that even before the separation of micro- and macronuclei as distinct structures the new synkaryon in this form is active in regeneration, and it is possible that in the case of Stentor, where no regeneration occurred, the essential stage in conjugation had been prevented.

Since micronuclear bodies after conjugation are able to form new macronuclei, the question arises as to whether a similar reconstruction may occur at other times. There is no evidence that during normal asexual reproduction any such process may take place when the macronucleus is artificially removed. Fragments from dividing Blepharisma containing only micronuclei undergo the same phenomena of differentiation and dedifferentiation as pieces lacking nuclear material entirely, and no indication of compensatory changes in the micronuclear bodies is apparent. This is of particular interest since there are several indications that Blepharisma, like Paramecium (Woodruff and Erdmann, '14), undergoes periodic nuclear reorganization during vegetative life.

SUMMARY

1. The two infusorians Spathidium spathula and Blepharisma undulans in the vegetative state possess a highly developed power of regeneration.

2. All fragments which regenerate both morphologically and physiologically contain both macro- and micronuclear material.

3. The process of regeneration is more direct in Blepharisma than in Spathidium.

a. In Blepharisma differentiated organelles are retained and made part of the new individual.

b. In Spathidium partial dedifferentiation of existing external structures precedes regeneration.

4. Fragments of Blepharisma derived from individuals in the earliest phases of division may restore their normal form in the absence of nuclear material of any sort. Such regenerated individuals, however, are unable to feed and gradually become completely dedifferentiated and disappear.

5. It is possible that formative substances liberated by the nucleus for the purpose of physiological regeneration are sufficient to complete the process when once begun.

6. The presence of micronuclei in such fragments does not affect their subsequent behavior.

7. Micronuclei alone during asexual reproduction are incapable of maintaining normal form or of giving rise to macronuclear material.

8. During conjugation complete regeneration in Spathidium is less frequent than during vegetative existence.

a. Fragments obtained after the beginning of micronuclear division and before the interchange of pronuclei fail to regenerate.

b. Pieces containing the synkaryon are capable of regenerating and dividing.

c. Conjugants cut soon after fusion or just prior to separation behave essentially like vegetative individuals.

d. Portions containing only resorbing macronuclear material and degenerating micronuclei may partially regain normal form but subsequently dedifferentiate and never divide.

9. Fragments obtained from starved individuals either fail to regenerate or become encysted after first passing through the processes of differentiation and dedifferentiation.

10. It has been impossible to produce an amicronucleate race by artificial means, and the evidence at hand indicates that idiochromatin is essential for normal asexual reproduction.

11. If this is true, it is highly probable that the nuclear apparatus of the recently described amicronucleate races of Infusoria represents an amphinucleus which has arisen through some irregularity during conjugation.

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STUDIES ON SPATHIDIUM SPATHULA

II. THE SIGNIFICANCE OF CONJUGATION

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TWENTY-FIVE FIGURES

CONTENTS

Introduction 133
Materials and methods 136
Endomixis and conjugation in Spathidium 138
A. Endomixis 138
B. Conjugation 138
Description of the cultures 142
A. Parent culture I 148
B. Series A 148
C. Series E 171
Results and discussion 178
A. The 'life-cycle' 178
B. The effects of conjugation on the division rate 181
1. General data 181
2. Initial effect 186
3. Effect in slowly and rapidly dividing cultures 188
4. Later effect of conjugation 188
C. The effect of conjugation on longevity 190
1. General survival value 190
2. Immediate survival value 191
3. Generations of cultures 191
4. Cumulative effects 192
D. Effect of close inbreeding 194
Summary 196

INTRODUCTION

The question of the significance of conjugation in the life-history of Infusoria has been the subject of discussion and experiment since the pioneer work of Bütschli[1] and Engel-

[1] Bütschli: Studien über die ersten Entwickelungsvorgänge der Eizelle, die Zelltheilung und die Konjugation der Infusorien. Abh. d. Senckenb. nat. Gesellsch., Frankfurt a. M., Bd. 10, 1876.

mann,[2] and the classic studies of Maupas.[3] For the purpose in hand, however, it is needless to reiterate the readily available history of the problem. The most recent evidence is as follows:

The study of pedigree cultures of various Infusoria has demonstrated that under favorable environmental conditions these organisms can dispense with conjugation and still continue to live and reproduce indefinitely.[4] Thus synkaryon formation is not an essential event in the life-history. However, another phenomenon, endomixis, occurs periodically in certain species, e.g., Paramecium aurelia and Paramecium caudatum.[5] This involves a reconstitution of the macronucleus of the cell from the micronucleus and thus exhibits one aspect of the nuclear reorganization which takes place in conjugation, but with the diagnostic event of conjugation—synkaryon formation—necessarily omitted because endomixis takes place within a single individual. Although the data at hand[6] indicate that in Paramecium aurelia and Paramecium caudatum suppression of endomixis results in death, a race of Paramecium calkinsi now long under culture in this laboratory has never undergone endomixis,[7] and the same is true of

[2] Engelmann: Ueber Entwickelung und Fortpflanzung von Infusorien. Morph. Jahrbuch, Bd. 1, 1876.

[3] Maupas: Recherches expérimentales sur la multiplication des Infusoires ciliés, Arch. d. Zool. exper. et gén., 2me sér., T. 6, 1888; Le rajeunissement karyogamique chez les Ciliés, ibid., T. 7, 1889.

[4] Woodruff: Two thousand generations of Paramecium. Arch. f. Protistenkunde, Bd. 21, 1911; The present status of the long-continued pedigree culture of Paramecium aurelia at Yale University, Proc. Nat. Acad. Sciences, vol, 7, 1921. Enriques: Della degenerazione senile negli Infusori. Atti R. Accad. Lincei Roma, vol. 14, 1905; Ancora della degenerazione senile negli Infusori, ibid; Duemila cinquecento generazioni in un Infusorio, senza coniugazione nè partenogenesi, nè depressioni, R. Accad. d. Scienze d. Instituto di Bologna, 1916. Baitsell: Experiments on the reproduction of hypotrichous Infusoria, Jour. Exp. Zoöl., vol. 16, 1914. Dawson: An experimental study of an amicronucleate Oxytricha, Part I, Jour. Exp. Zoöl., vol. 29, 1919; Part II, ibid., vol. 30, 1920.

[5] Woodruff and Erdmann: A normal periodic reorganization process (endomixis) without cell fusion in Paramecium. Jour. Exp. Zoöl., vol. 17, 1914. Erdmann and Woodruff: The periodic reorganization process in Paramecium caudatum, ibid., vol. 20, 1916.

[6] Woodruff: The influence of general environmental conditions on the periodicity of endomixis in Paramecium aurelia, Biological Bulletin, vol. 33, 1917.

[7] Spencer: In press.

certain other ciliates studied. Thus it is clear that endomixis does not occur in some forms during free life, and in these neither endomixis nor conjugation is essential for continued existence.

Other studies have been directed more particularly to determine what effect conjugation produces when it does occur. Jennings' extensive experiments[8] on Paramecium aurelia and Paramecium caudatum lead him to conclude that the significant result of conjugation is the production of variations and that conjugation is not attended by a stimulation or 'rejuvenation' of the organism. Calkins,[9] on the other hand, from his long-continued attack on the problem, culminating in recent experiments on Uroleptus mobilis, admits that variability following conjugation is probably a fact, but puts emphasis on inherent senescence in the absence of endomixis or conjugation and demonstrates that an increase of vitality is a most significant result of conjugation.

The present investigation is an intensive study of the significance of fertilization in the life-history of Spathidium spathula, with special reference to the question of the relation of this phenomenon to reproductive activity and longevity of the organism. In brief: Does conjugation increase the reproductive rate?

Spathidium spathula was selected, after long search for favorable material, as the subject of the experiments, because it affords the several features which are essential for the successful attainment of the desired data: 1) sufficient size to render practicable the daily isolation of large numbers of animals; 2) non-occurrence of endomixis in free-living stages, and, most important of all, 3) the frequent onset of epidemics

[8] Jennings: Age, death, and conjugation in the light of work on lower organisms, Pop. Sci. Monthly, 1912; The effect of conjugation in Paramecium, Jour. Exp. Zoöl., vol. 14, 1913; Life and death, heredity and evolution, in unicellular organisms, Boston, 1920.

[9] Calkins: Protozoology, Philadelphia, 1909; Cycles and rhythms and the problem of 'immortality' in Paramecium, Amer. Nat., vol. 49, 1915; Uroleptus mobilis Engelm., II. Renewal of vitality through conjugation, Jour. Exp. Zoöl., vol. 29, 1919; III. A study of vitality, ibid., vol. 31, 1920.

of conjugation between pedigree animals so that non-conjugant and conjugant lines can be secured and compared especially at crucial periods.

A study of the structure, classification, and behavior of Spathidium spathula has already been published in part I of these studies, and the reader is referred to this for a further introduction to the problem at hand. Summaries of the earlier results of the present study have also appeared.[10]

MATERIALS AND METHODS

The specimen of Spathidium spathula which was the progenitor of all the animals employed in these studies was found on November 5, 1920, in a jar of decaying vegetable débris collected at New Haven. This individual was isolated on a depression slide and provided with food in two drops of standard beef extract.[11]

A large quantity of the extract was made up, distributed in test-tubes plugged with cotton, and sterilized in an autoclave. This afforded a uniform basic culture fluid for all the experiments. Since Spathidium is a carnivorous form which feeds almost exclusively on small ciliates, it was necessary to add these organisms to the beef extract. Accordingly, several flasks of timothy-hay infusions were seeded with Colpidium at the start of the work and from time to time fresh flasks were inoculated so that, in so far as possible, the actual food supply remained constant throughout. It was the practice daily to take a small amount of material from several flasks, mix it in a watch-glass, and then add one drop of this Colpidium culture to the beef-extract medium on each slide. The bacteria which developed in the flasks provided food for the

[10] Woodruff and Spencer: Studies on Spathidium spathula, I. The structure and behavior of Spathidium with special reference to the capture and ingestion of its prey, Jour. Exp. Zoöl., vol. 35, 1922; The early effects of conjugation on the division rate of Spathidium spathula, Proc. Soc. for Exper. Biol. and Med., vol. 18, 1921; The survival value of conjugation in the life-history of Spathidium spathula, ibid.

[11] Woodruff and Baitsell: The reproduction of Paramecium aurelia in a 'constant' culture medium of beef extract, Jour. Exp. Zoöl., vol. 11, 1911.

Colpidia, and the latter in turn afforded the diet of the Spathidia.

For comparison a special set of eleven cultures was conducted in which malted milk was substituted for beef extract as the basic culture fluid for Spathidium. No essential difference in the results was obtained, so the cultures were stopped after fifteen days.

The depression slides with culture media containing the pedigree Spathidia were kept in large moist chambers at room temperature. A record of the temperature was kept by a thermograph. Daily isolations of an animal from each line were made under a low-power Zeiss binocular microscope and the number of divisions recorded. From time to time specimens were preserved for cytological study.

The general modus operandi of conducting the pedigree lines was, briefly stated, as follows: From the original animal four lines were carried as the stem culture (designated I). Animals left over at the daily isolation were kept as stock, or allowed to conjugate or encyst. From time to time pairs of conjugants were isolated and their progeny established as 'new' pedigree groups of cultures from the stem line (I) or its derivatives. The first group so instituted was designated A, the second B, etc. Usually these groups comprised the progeny of two pairs of conjugants. The cultures derived from one pair were designated a and b, respectively, and those from the other pair, c and d. In a few cases the progeny of other pairs were carried and designated e and f, etc. In every instance the culture derived from a single ex-conjugant comprised four lines of animals (for example, culture Aa lines 1, 2, 3, and 4) and the average daily division rate of these four lines, again averaged for five-days periods, forms the basic data of the present study. At certain periods nearly 200 isolations were made daily, so that the investigation has involved the manipulation of upward of 100,000 pedigree animals (cf. fig. 7 and table 1).

It should, perhaps, be emphasized that all the groups of cultures, which are directly compared for crucial changes in

the division rate following conjugation, were, of course, living synchronously, and were supplied with identical culture medium and subjected to the same temperature variations.

ENDOMIXIS AND CONJUGATION IN SPATHIDIUM

Since there were no data extant in regard to either endomixis or conjugation in Spathidium, it was necessary to determine whether the former occurs, and the essential features of the latter process.

A. Endomixis

A careful cytological examination of a long series of animals preserved during the life of one of the pedigree cultures, and of special animals at certain periods in others, gave no evidence of endomixis, and it is certain that this phenomenon does not occur in this race of Spathidium during active life. Therefore, if endomixis occurs it must be during encystment, and, as a matter of fact, Miss Moore in this laboratory investigated the question in this race of Spathidium and found endomixis during encystment.[12]

Thus this complicating factor in the study of the life-history of P. aurelia and P. caudatum plays no part in the results discussed in the present paper on Spathidium, and the ground is clear for a concentration of the attack on conjugation.

B. Conjugation

Epidemics of conjugation could be secured almost at will by allowing a few animals to remain on a slide for a day or two without an adequate supply of food and, as already mentioned, it was chiefly for this reason that Spathidium was selected for the present investigation. However, in this regard, this race of Spathidium is quite different from the one studied by Moody,[13] which gave no evidence of ability to con-

[12] Moore: Endomixis and encystment in Spathidium spathula, Proc. Soc. Exp. Biol. and Med., vol. 19, 1922.

[13] Moody. Observations on the life-history of two rare ciliates, Spathidium spathula and Actinobolus radians. Jour. Morph., vol. 23, 1912.

jugate. As we have pointed out,[14] Moody's animals apparently represent an amicronucleate race, which obviously accounts for their failure to conjugate.

The first conjugants were secured in the present study on November 12, 1920, from animals taken from culture I at the thirtieth generation. Ten pairs were isolated, and on the following day all were exconjugants. The four exconjugants from two pairs were the progenitors of cultures Aa, Ab, Ac, and Ad. The remainder were carried in pedigree culture for a few days, to supply specimens for cytological study, etc. In this series of twenty exconjugants only one died without dividing, and this high viability is characteristic of the long series of exconjugants studied in starting the various subcultures during the experiments.

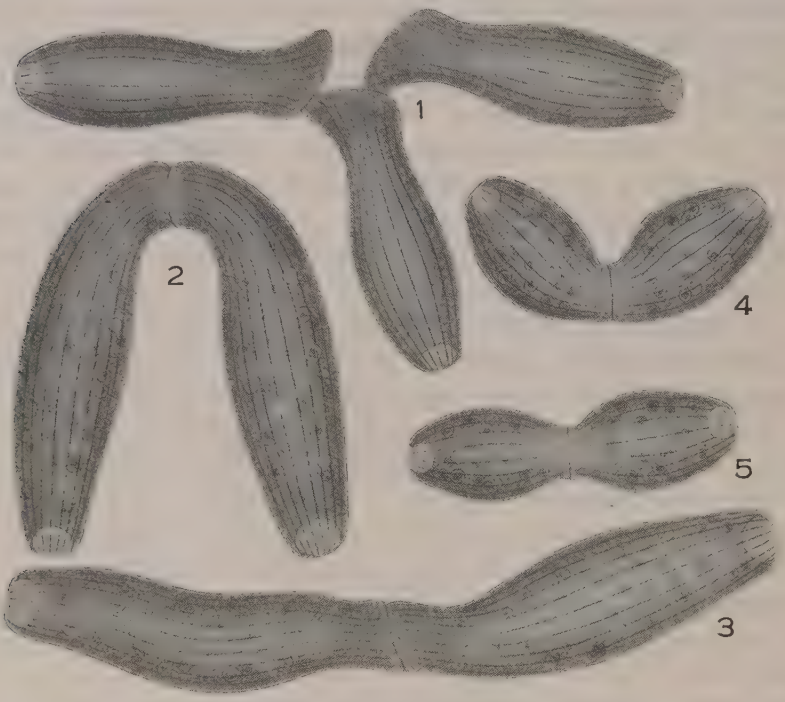
Animals ready to conjugate are essentially typical in form, though frequently somewhat more slender and transparent than well-fed individuals, and thus resemble specimens about to encyst.[15] Apparently the conditions leading to encystment and to conjugation are quite similar, because both phenomena often occur simultaneously in a single slide culture. Since, as Moore has shown, endomixis is undergone during encystment, it seems clear that both conjugation and endomixis represent, in general, different ways of meeting the same exigencies. However, since encystment will take place immediately after conjugation, while an interval of about two weeks usually intervenes before the cells of a culture show a strong tendency to conjugate again, it seems natural to assume that endomixis does not invariably occur during encystment, and this has been substantiated by Moore's results.

Animals ready to conjugate produce an effect on neighboring individuals which recalls the remarkable behavior of Spathidium in relation to its paralyzed prey[16]—the peristome regions of the Spathidia exhibiting a tendency to come in contact. Frequently, at such times, several individuals will

[14] Studies on Spathidium, I. Loc. cit., p. 196.
[15] Cf. Studies on Spathidium, I. Loc. cit., plate.
[16] Studies on Spathidium, I. Loc. cit., p. 201.

become organically connected so that bizarre groups result (fig. 1).

When normal peristomal contact has been established, the two animals shift and turn until the peristomal edges are exactly in apposition, and then endoplasmic fusion occurs.



Figs. 1 to 5 Fig. 1, an example of the bizarre groups of organically connected Spathidia which occur during epidemics of conjugation. Figs. 2 and 3, early conjugants. Figs. 4 and 5, later conjugants.

Usually this is accomplished within fifteen minutes and the animals present the appearance shown in figures 2 and 3. Then gradually the conjugants become smaller and more spherical (figs. 4 and 5), the maximum change in size and form being attained in about eight or ten hours, and maintained for about the same length of time when separation occurs. Thus at room temperature the animals remain paired from sixteen to twenty hours. The exconjugants retain for

an hour or so the rounded form of conjugants, but their future history depends entirely upon the food supply. Unless Colpidia or similar ciliates are present, the Spathidia become greatly elongated, tiny, transparent cells and invariably die within twenty-four hours, except in the few cases where encystment takes place. When Colpidia are available, the Spathidia feed rapidly, become greatly distended with food, and divide usually within twenty-four hours.

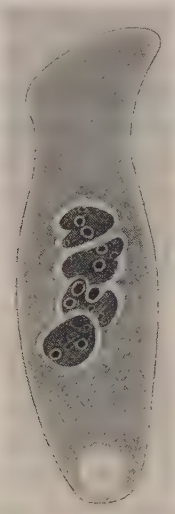


Fig. 6 Exconjugant showing the four macronuclear anlagen and several micronuclei.

The fact that exconjugants quickly die unless fed, and that animals which have been fed are distorted with the remains of ingested Colpidia in various stages of disintegration, render especially difficult the study of the cytological phenomena involved in conjugation. For the purpose of the present paper it is sufficient to state that typically four clearly delineated macronuclear anlagen and several micronuclei are present in each conjugant before separation and in each exconjugant (fig. 6) before it divides. Usually by this time there are only traces left of the old macronuclear material. The first division of the exconjugant distributes two anlagen

to each daughter cell, and after the next division the animals exhibit the typical vermiform macronuclear apparatus accompanied by from five to eight micronuclei.

DESCRIPTION OF THE CULTURES

A general survey of the various groups is afforded by the pedigree chart (fig. 7) and synoptic table (table 1).[17] The latter gives for each culture, in parallel columns, its designation letters, source, calendar duration, duration in five-day periods, generations attained, and fate.

The initial date given for each of the cultures (except I) is that on which the progenitor became an exconjugant, but the computing of the five-day periods does not start until after the exconjugant divided. Usually the number of days' duration of a culture has not, of course, been an exact multiple of five, and therefore the numbers in the 'period column' represent the five-day period in which the respective cultures were terminated.

The parent culture, I, was started with the original progenitor of all the animals employed in these studies, and is itself directly or indirectly the parent culture of all the series. A study of figure 7 and table 1 shows that culture I is the immediate progenitor of two groups of cultures, A and E, which in turn are the progenitors, respectively, of two series of cultures—series A and series E; series A comprising twenty-seven groups and series E, eight groups. Accordingly, the description and discussion of the results will be simplified by considering first culture I and group A, and then the derivatives of A (pp. 148 to 171), and passing finally to group E and its derivatives (pp. 171 to 177). Since the results derived from I, A, and derivatives (A series) are in entire accord with those from E and its derivatives (E series), especial emphasis will be placed on the former. As a matter of fact, the data derived from culture I and cultures Aa, Ab, Ac, Ad, give a clear-cut and unequivocal result to which the abundant concordant evidence from all the rest but contributes.

[17] It is assumed that the reader will refer to this figure and table when reading each of the descriptions of the cultures which follow.

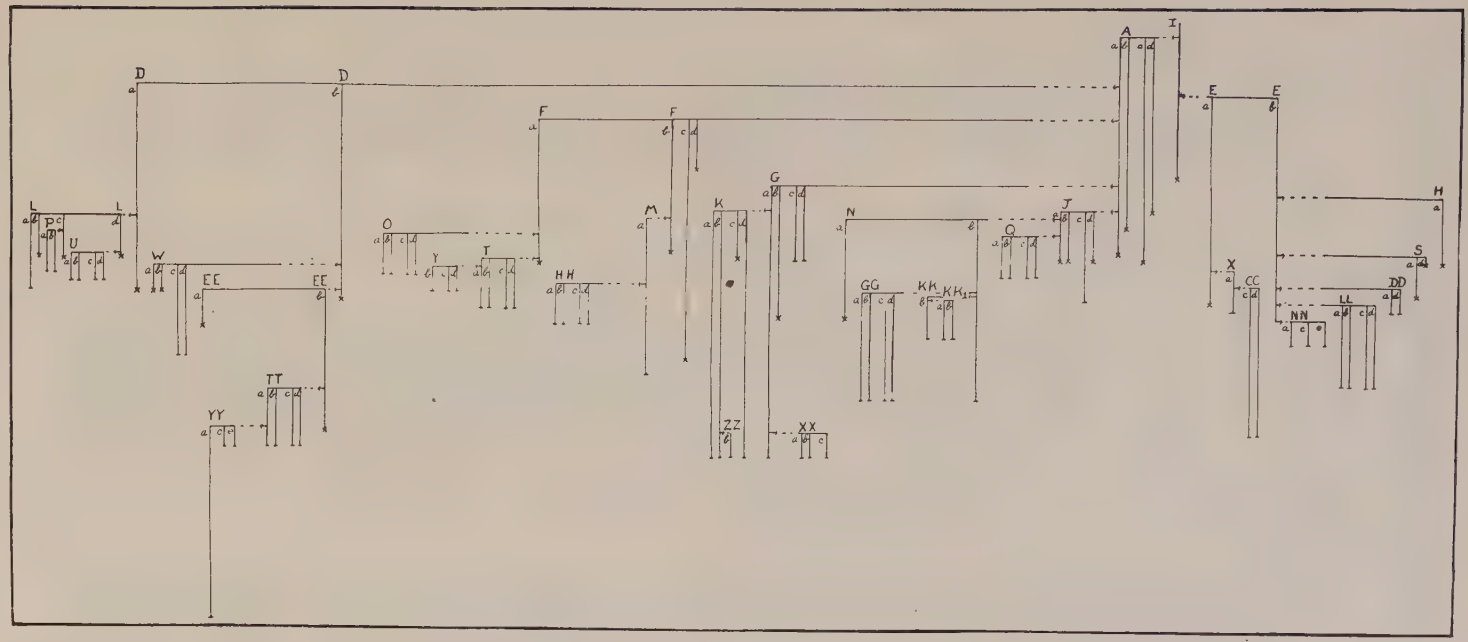


Fig. 7 Synoptic pedigree chart of culture I and its derivatives. Series A to the left, series E to the right. Length of vertical lines represent relative duration of respective cultures; —, stopped; X, died.

TABLE 1

Synopsis of the cultures employed

DESIGNATION OF CULTURE	ORIGIN: CULTURE AND DIVISION	DURATION IN DAYS	DURATION IN PERIODS	DURATION IN DIVISIONS	FATE: DIED OR STOPPED	TEXT FIGURE NUMBER
		1920-1921				
I	'wild'	Nov. 5–Feb. 11	19	234	d	8
Aa	I– 30	Nov. 13–Mar. 27	26	338	d	8
Ab	I– 30	Nov. 13–Mar. 14	23	268	d	
Ac	I– 30	Nov. 13–Apr. 3	27	347	d	
Ad	I– 30	Nov. 13–Mar. 4	21	253	d	
Ba	I– 99	Dec. 3–Dec. 12	2	17	s	
Bb	I– 99	Dec. 3–Dec. 11	2	16	d	
Ca	I–104	(died without dividing)				
Cb	I–104	(died without dividing)				
Da	Aa–104	Dec. 10–Apr. 15	25	272	d	9
Db	Aa–104	Dec. 10–Apr. 26	27	291	d	
Ea	I–146	Dec. 17–Apr. 19	25	211	s	24
Eb	I–146	Dec. 17–May 9	29	267	d	
Fa	Aa–178	Jan. 1–Mar. 29	17	224	d	15
Fb	Aa–178	Jan. 1–Mar. 27	16	169	d	
Fc	Aa–178	Jan. 1–May 29	29	293	d	
Fd	Aa–178	Jan. 1–Feb. 1	6	96	d	
Ga	Aa–267	Feb. 9–July 31	33	546	s	18 a
Gb	Aa–267	Feb. 9–May 8	16	209	d	and
Gc	Aa–267	Feb. 9–Mar. 26	9	109	d	18 b
Gd	Aa–267	Feb. 9–Mar. 26	9	108	d	
Ha	Eb– 98	Feb. 12–Mar. 28	8	107	d	24
Hb	Eb– 98	(died without dividing)				
Ja	Aa–295	Feb. 24–Mar. 26	6	78	d	20
Jb	Aa–295	Feb. 24–Mar. 26	6	81	d	
Jc	Aa–295	Feb. 24–May 2	11	177	s	
Jd	Aa–295	Feb. 24–Mar. 26	6	77	d	
Ka	Ga– 43	Feb. 26–July 31	30	210	s	19
Kb	Ga– 43	Feb. 26–July 31	30	235	s	
Kc	Ga– 43	Feb. 26–Mar. 28	6	71	d	
Kd	Ga– 43	Feb. 26–July 31	30	460	s	

TABLE 1—*Continued*

DESIGNATION OF CULTURE	ORIGIN: CULTURE AND DIVISION	DURATION IN DAYS	DURATION IN PERIODS	DURATION IN DIVISIONS	FATE: DIED OR STOPPED	TEXT FIGURE NUMBER
		1921				
La	Da–205	Feb. 28–Apr. 24	9	148	s	11
Lb	Da–205	Feb. 28–Mar. 27	5	70	d	
Lc	Da–205	Feb. 28–Mar. 28	5	70	d	
Ld	Da–205	Feb. 28–Mar. 29	5	69	d	
Ma	Fb–131	Mar. 3–June 11	19	260	s	17
Mb	Fb–131	(died without dividing)				
Na	Ja– 25	Mar. 6–May 8	12	155	s	21 and
Nb	Ja– 25	Mar. 6–June 26	22	301	s	22
Oa	Fa–179	Mar. 11–Apr. 7	5	78	s	15
Ob	Fa–179	Mar. 11–Apr. 7	5	77	s	
Oc	Fa–179	Mar. 11–Apr. 7	5	78	s	
Od	Fa–179	Mar. 11–Apr. 7	5	76	s	
Pa	Lc– 27	Mar. 11–Apr. 7	5	67	s	11
Pb	Lc– 27	Mar. 11–Apr. 7	5	68	s	
Qa	Ja– 42	Mar. 11–Apr. 7	5	72	s	23
Qb	Ja– 42	Mar. 11–Apr. 7	5	76	s	
Qc	Ja– 42	Mar. 11–Apr. 7	5	80	s	
Qd	Ja– 42	Mar. 11–May 11	11	168	s	
Ra	Ac–238	(died without dividing)				
Rb	Ac–238	(died without dividing)				
Sa	Eb–160	Mar. 8–Apr. 18	6	45	d	
Sb	Eb–160	(died without dividing)				
Sc	Eb–160	(died without dividing)				
Sd	Eb–160	Mar. 18–Mar. 29	2	33	d	
Ta	Fa–224	Mar. 31–May 2	6	95	s	15
Tb	Fa–224	Mar. 31–May 2	6	96	s	
Tc	Fa–224	Mar. 31–May 2	6	94	s	
Td	Fa–224	Mar. 31–May 2	6	91	s	
Ua	Ld– 69	Mar. 31–Apr. 17	3	49	s	12
Ub	Ld– 69	Mar. 31–Apr. 17	3	50	s	
Uc	Ld– 69	Mar. 31–Apr. 17	3	49	s	
Ud	Ld– 69	Mar. 31–Apr. 17	3	53	s	

TABLE 1—*Continued*

DESIGNATION OF CULTURE	ORIGIN: CULTURE AND DIVISION	DURATION IN DAYS	DURATION IN PERIODS	DURATION IN DIVISIONS	FATE: DIED OR STOPPED	TEXT FIGURE NUMBER
		1921				
Wa	Db–270	Apr. 1–Apr. 17	3	45	d	13
Wb	Db–270	Apr. 1–Apr. 17	3	45	d	
Wc	Db–270	Apr. 1–May 27	11	127	s	
Wd	Db–270	Apr. 1–May 27	11	113	s	
Xa	Ea–175	Apr. 1–Apr. 27	5	71	s	24
Xb	Ea–175	(exconjugant lost)				
Ya	Ta– 16	Apr. 6–Apr. 7				
Yb	Ta– 16	Apr. 6–Apr. 21	3	40	s	16
Yc	Ta– 16	Apr. 6–Apr. 21	3	45	s	
Yd	Ta– 16	Apr. 6–Apr. 21	3	47	s	
CCa	Xa– 34	(not fed–encysted)				
CCb	Xa– 34	(not fed–encysted)				
CCc	Xa– 34	Apr. 13–July 16	18	305	s	
CCd	Xa– 34	Apr. 13–July 16	18	311	s	
DDa	Eb–226	Apr. 17–May 2	3	49	s	25
DDb	Eb–226	(died without dividing)				
DDc	Eb–226	(died without dividing)				
DDd	Eb–226	Apr. 17–May 2	3	41	s	
EEa	Db–289	Apr. 19–May 14	4	51	d	14
EEb	Db–289	Apr. 19–July 24	17	171	d	
GGa	Nb–122	Apr. 22–June 26	13	202	s	22
GGb	Nb–122	Apr. 22–June 26	13	190	s	
GGc	Nb–122	Apr. 22–June 26	13	179	s	
GGd	Nb–122	Apr. 22–June 26	13	190	s	
HHa	Ma–106	Apr. 22–May 17	5	77	s	17
HHb	Ma–106	Apr. 22–May 17	5	81	s	
HHc	Ma–106	Apr. 22–May 17	5	82	s	
HHd	Ma–106	Apr. 22–May 17	5	82	s	
KKa	Nb–122	Apr. 22–(discarded)				
KKb	Nb–122	Apr. 22–May 17	5	69	s	21
KK_1a	KKb– 3	Apr. 23–May 17	5	79	s	
KK_1b	KKb– 3	Apr. 23–May 17	5	78	s	

TABLE 1—*Continued*

DESIGNATION OF CULTURE	ORIGIN: CULTURE AND DIVISION	DURATION IN DAYS	DURATION IN PERIODS	DURATION IN DIVISIONS	FATE: DIED OR STOPPED	TEXT FIGURE NUMBER
		1921				
LLa	Eb–236	Apr. 23–June 16	10	166	s	25
LLb	Eb–236	Apr. 23–June 16	10	172	s	
LLc	Eb–236	Apr. 23–June 16	10	177	s	
LLd	Eb–236	Apr. 23–June 16	10	167	s	
NNa	Eb–267	May 11–May 27	3	53	s	25
NNb	Eb–267	(died without dividing)				
NNc	Eb–267	May 11–May 27	3	57	s	
NNd	Eb–267	(died without dividing)				
NNe	Eb–267	May 11–May 27	3	55	s	
NNf	Eb–267	(died without dividing)				
TTa	EEb–150	June 26–July 31	7	127	s	14
TTb	EEb–150	June 26–July 31	7	130	s	
TTc	EEb–150	June 26–July 31	7	111	s	
TTd	EEb–150	June 26–July 31	7	131	s	
XXa	Ga–491	July 17–July 31	3	65	s	18 b
XXb	Ga–491	July 17–July 31	3	64	s	
XXc	Ga–491	July 17–July 31	3	60	s	
XXd	Ga–491	(died without dividing)				
YYa	TTa– 80	July 17–Nov. 5	23	300	d	14
YYb	TTa– 80	(died without dividing)				
YYc	TTa– 80	July 17–July 31	3	59	s	
YYd	TTa– 80	(died without dividing)				
YYe	TTa– 80	July 17–July 31	3	60	s	
YYf	TTa– 80	(died without dividing)				
ZZa	Kb–217	(died without dividing)				
ZZb	Kb–217	July 17–July 31	3	32	s	19

A. Parent culture I

With the data presented by figure 7 and table 1, in regard to culture I, in mind, a glance at figure 8 (solid line) shows that this culture during the first five-day period reproduced at an average rate of somewhat over four divisions a day—a high point which it never again attained. From this point the trend of the rate is gradually and, excepting certain fluctuations, consistently downward until the culture died at the end of ninety-five days (nineteen periods) at the 234th generation.

B. Series A

1. Group A. As already stated, cultures Aa and Ab were started with the exconjugants of a pair from culture I, and cultures Ac and Ad from another pair from culture I on November 13, 1920 (fig. 7 and table 1). An examination of figure 8 shows:

1. A gradual downward trend in the division rate of all the A cultures from start to end by death, similar to that exhibited by culture I.

2. By the end of the third period (fifteen days) all of the A cultures were dividing more rapidly than culture I, and this rate was maintained consistently (with the sole exception of Ac and Ad during period 14) throughout the life of culture I. During the eighteen periods when culture I and the A cultures were running synchronously, culture I divided 204 times, Aa 275 times, Ab 236 times, Ac 217 times, and Ad 242 times. In brief, the progeny of exconjugants divided more rapidly than the progeny of sister cells which had not conjugated.

3. All the A cultures survived culture I. Aa survived 9 periods and divided 63 times; Ab, 7 periods and divided 32 times; Ac, 11 periods and divided 130 times, and Ad, 4 periods and divided 11 times after the death of I. The progeny of exconjugants possessed the power to live longer and divide more times than the progeny of sister cells which did not conjugate.

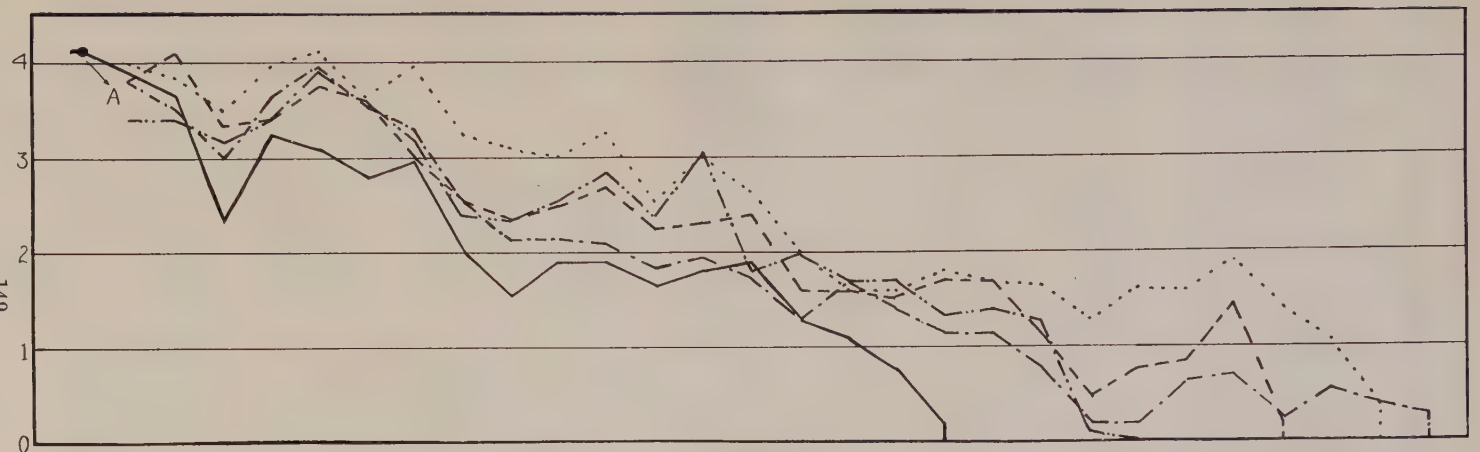


Fig. 8 Graph of the division rate of culture I and group A. Ordinates indicate the average daily division rate of the four lines of each culture, again averaged for each five-day period. Periods expressed by the abscissa. •, origin of exconjugant cultures; culture line terminating on abscissa = death; otherwise = stopped.
Culture I, —; Aa, . . . ; Ab, – – – –; Ac, –.–; Ad, –. .–.

The data from this experiment indicate that, following conjugation, the reproductive rate and longevity are increased, thus implying that all the processes of the cell, including reproduction, proceed with greater vigor.

We may now proceed to an accumulation of evidence from similar experiments.

2. Groups B and C. The progeny of a single pair of conjugants from culture I designated Ba and Bb, respectively, were carried for two periods. Ba died at this time, and therefore Bb was discontinued. Similarly, Ca and Cb were started with exconjugants of a pair of conjugants from I, but both died without dividing. That there is no special significance attached to these results will be obvious as we proceed. The exceedingly high viability of exconjugants is demonstrated by the very few exceptions, of which the B and C cultures are examples.

3. Group D. Da and Db were started as exconjugants from a pair from Aa. Designating culture I as the parental generation, the A cultures represent the F_1 generation and the D cultures, the F_2 generation. An examination of figure 9 shows:

1. A gradual downward trend of the division rate of Da and Db from start to end by death.

2. During the first ten periods Da and Db divided less rapidly than Aa, but shortly thereafter both divided more rapidly than Aa and consistently maintained this higher rate during the eleven remaining periods of the life of Aa. During the twenty-one periods when Aa and the D group was running synchronously, culture Aa divided 234 times, Da 260 times, and Db 263 times. Moreover, since Aa during this period, as we have seen, exceeded culture I in rate of division, it is clear that the D group also exceeded culture I. The comparison of the data from culture Aa and the D group thus corroborates the similar data from culture I and the A group; the only difference being that the division rate of the A group exceeded the parent culture I, almost from the start, while that of the D group did not exceed the parent culture Aa

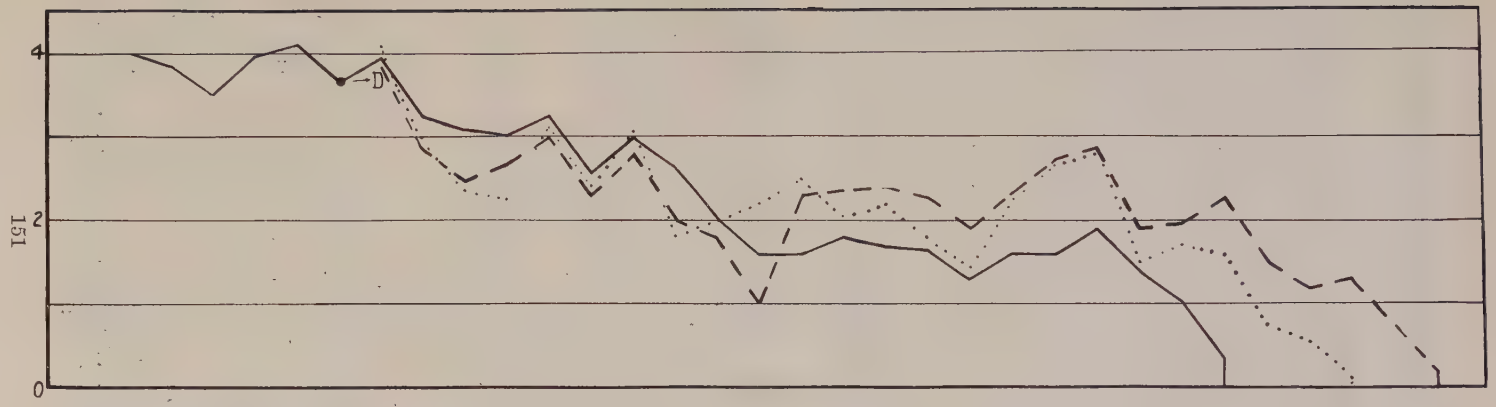


Fig. 9 Graph of the division rate of group D from culture Aa. Aa, —; Da, ...; Db, – – – –.
(For other details of plotting, cf. fig. 8.)

until about the middle of the experiment. However, the significant result—increase in the average division rate of the exconjugant group during the synchronous life of the parent culture—is the same in both series of experiments.

3. Both the D cultures survived their parent culture (Aa) as well as all of the A group of cultures and therefore, of course, the stem culture I. Da survived Aa 4 periods and divided 12 times and Db survived 6 periods and divided 28 times after the death of Aa. These data agree with those derived from culture I and the A groups—the progeny of exconjugants possess greater longevity and divide more times than the progeny of sister cells which did not conjugate.

4. Group L. The progeny of two pairs of conjugants from Da constitute cultures La, Lb, Lc, and Ld, which are the F_3 generation with reference to the stem culture (I). Figure 10 shows:

1. La consistently maintained from the start a higher rate of division than Da to the death of the latter. While they were running synchronously, Da divided 63 times and La 127 times. Lb, Lc, and Ld also divided more rapidly than Da, but all three suddenly died during the fifth period, when the division rate was high, apparently from some accidental cause since quite a number of other cultures in different experiments succumbed at the same time.

Aside from this abrupt termination of Lb, Lc, and Ld, all the data from the L cultures are in accord with that derived from those already discussed.

5. Group P. These cultures were started with a pair of exconjugants from Lc and represent the F_4 generation from culture I through Aa (F_1), Da (F_2), and Lc (F_3). The initial division rate of both P cultures was slightly lower than that of the parent culture, and thus affords an example of decreased reproductive activity following conjugation. The P group was discontinued at the end of five periods, because, as already stated, culture Lc suddenly died. Accordingly, the results of the experiment are inconclusive in regard to other points, but it is significant that both of the P cultures

were consistently increasing in division rate when discontinued (fig. 11).

6. Group U. Ua, Ub, Uc, and Ud represent the progeny of two pairs of conjugants from one-day stock of Ld, taken the day this culture suddenly died when dividing nearly three times daily (table 1 and figs. 7, 10, and 12). The cultures represent the F_4 generation. Figure 12 shows:

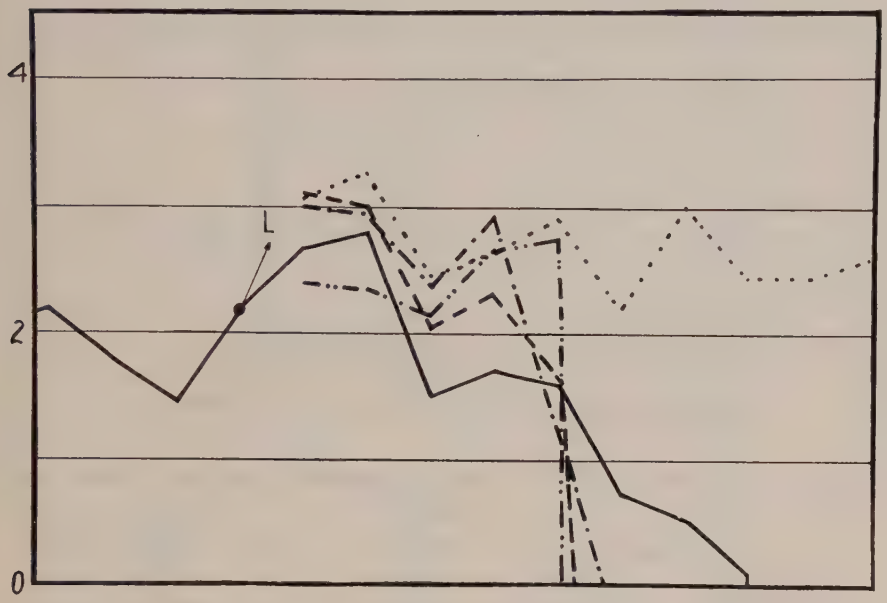


Fig. 10 Graph of the division rate of group L from culture Da. Da, — ; La, . . . ; Lb, – – – ; Lc, –.–; Ld, –. .–.

1. An initial acceleration of the division rate of all the U cultures to a point higher than that ever attained by the parent Ld culture.

2. There are no data in regard to longevity owing to the death of the parent culture. The U group therefore was stopped at the end of three periods.

7. Group W. We now turn from the derivatives of culture Da to those of Db, the first of which are Wa, Wb, Wc, and Wd. This group arose as exconjugants from two pairs, and

represent the F_3 generation from culture I. A study of figure 13 shows:

1. A remarkable increase in division rate in all the exconjugant cultures during the first three periods.

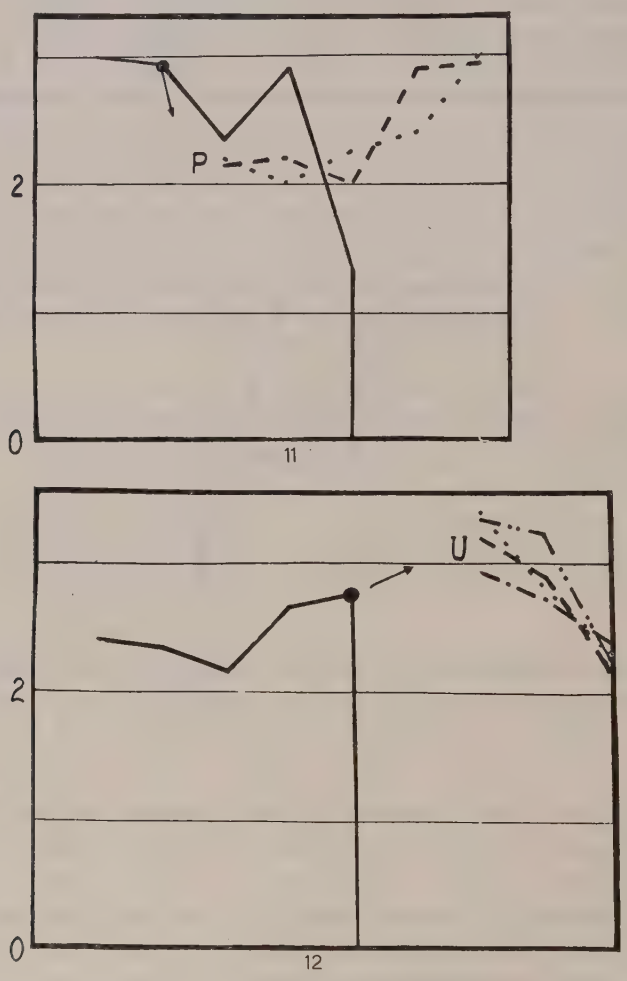


Fig. 11 Graph of the division rate of group P from culture Lc. Lc, —; Pa, . . .; Pb, – – –.

Fig. 12 Graph of the division rate of group U from culture Ld. Ld, —; Ua, ...; Ub, – – –; Uc, –.–; Ud, –. .–.

2. Cultures Wa and Wb suddenly died out when dividing rapidly during the fourth period. This is probably of no significance, since several cultures in other experiments succumbed at this time.

3. Cultures Wc and Wd, however, continued to live long after the parent Db culture died, and finally were stopped at the end of period 11.

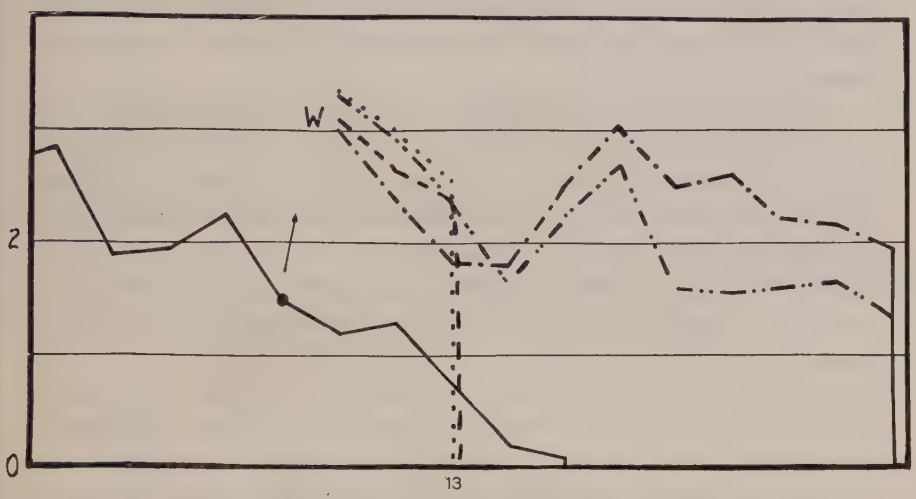


Fig. 13 Graph of the division rate of group W from culture Db. Db, — ; Wa, . . . ; Wb, – – – ; Wc, –.– ; Wd, –. .–.

8. Group EE. This group comprises two cultures (EEa and EEb), again representing the F_3 generation from culture I through Aa and Db, and arising from the latter culture just as it was dying out. The following conclusions are obvious from the data presented in figure 14:

1. Markedly higher division rate in both EEa and EEb than in the parent culture Db for the five periods before its death.

2. Culture EEa suddenly died when dividing rapidly at the end of its fourth period. EEb, however, was carried for thirteen periods longer when it died at the 171st generation.

9. Group TT. The progeny of two pairs of exconjugants from culture EEb constitute cultures TTa, TTb, TTc, and TTd; the F_4 generation from the stem culture I. The group was carried for seven periods and then stopped. Figure 14 presents data which justify the following statements:

1. A remarkable acceleration in division rate occurred immediately in all the exconjugant cultures, and this high rate was maintained until the experiment was discontinued. The rate averages above three divisions per day, which is greater than that shown by its parent culture (EEb) and its grandparental culture (Db).

2. There was every indication, when the experiment was discontinued two periods after the death of EEb, that the TT group could be carried on indefinitely. Obviously, the life of this pedigree race of Spathidium, from culture I through Aa, Db, and EEb, was prolonged by the TT cultures of the F_4 generation. All earlier generations (parent to F_3) had died, and the gradually waning division rate was restored to a high level.

10. Group YY. It has been seen that the TT group exceeded in division rate its ancestors for three generations. Although a stimulation of the rate followed the conjugation which initiated each culture, nevertheless the gain was never so great as the loss in vitality during the previous generation. In short, the average division rate at start of the A group, which was four divisions per day, had never since been attained by the cultures derived from it. An attempt to 'build up' the rate was therefore made by starting exconjugant cultures from the TTa culture when it was dividing rapidly, i.e., about three and one-half times per day on the average. These new cultures, designated YYa, YYc, and YYe comprised the progeny of a single member of each of three pairs of conjugants, and represent the F_5 generation from the stem culture I. From a study of figure 14 and reference to figure 9 the following facts are clear:

1. The division rate of the three YY cultures averaged considerably higher than that of TTa for the three periods dur-

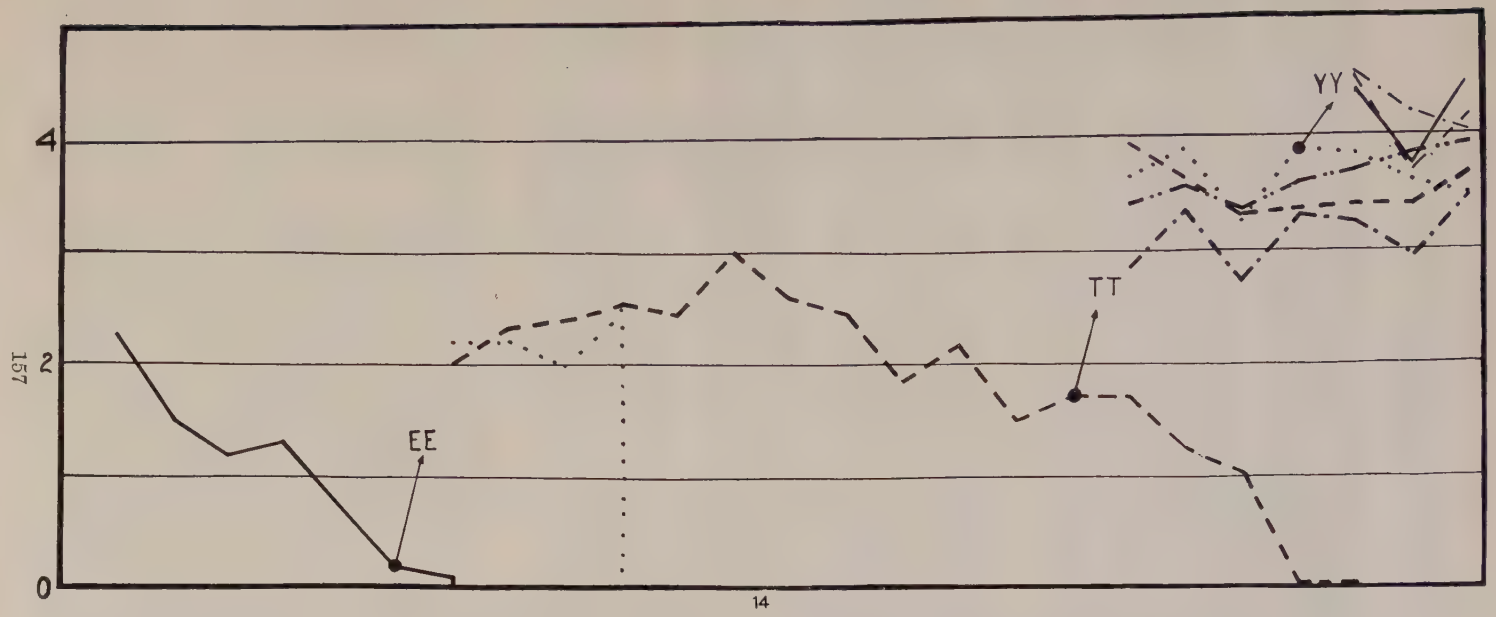


Fig. 14 Graph of the division rate of group EE from culture Db, group TT from culture EEb, and group YY from culture TTa. Db, — ; (in EE group) EEa, ... ; EEb, – – – – ; (in TT group) TTa, ... ; TTb, – – – – ; TTc, –.– TTd, –. .–; (in YY group) YYa, — ; YYc, – – – ; YYe, –.–.

ing which the experiment was continued; a rate actually higher than that ever exhibited by any of the ancestral cultures. YYa at its third period averaged almost four and one-half divisions per day.

2. Making due allowance for a slight increase in temperature at this period, it is still clear that the initial vitality, as indicated by reproductive rate, of the pedigree race has been restored.

3. YYa was continued until it died at the 300th generation after twenty-three periods under culture (omitted from fig. 14).

But, and this is highly significant, the pedigree race was continued during the following academic year (1921–1922) by progeny through conjugation from YYa—the F_6, F_7, etc., generations supplying the material studied by Miss Moore in this laboratory.

11. Group F. We have completed the description of the groups of cultures derived from Aa through D to the F_5 generation, and turn now to the F group and its derivatives (fig. 7 and table 1).

The F group was started with the progeny of two pairs of exconjugants from Aa. An examination of figure 15 shows:

1. All the F cultures started off at and maintained a slightly higher division rate than the parent culture (Aa) for six periods.

2. After the sixth period the higher division rate of the exconjugant lines was, in general, maintained, but, from unknown cause, Fd suddenly died when dividing rapidly at period 6, and Fa (O group, fig. 15) did the same at period 17, while Fb succumbed at period 16 after a somewhat gradual decline in vitality. In brief, three cultures of the F group died before, or essentially at, the time of death of the Aa culture. Fc, however, continued to prosper for twelve periods after the death of Aa, dying at the 293rd generation.

3. The results from group F, therefore, are definite in regard to an initial increase in division rate following conjugation, but in three out of four cultures are negative in regard to longevity.

12. Group O. As already stated, Fa died suddenly from unknown cause when it was dividing rapidly. Three periods before this happened, group O was started from Fa with the progeny of two pairs of conjugants and constitutes the F_3 generation from culture I through Aa and Fa. It was carried for five periods and then stopped, since the parent culture had died. The data from group O given in figure 15 show:

1. A marked increase in the division rate of all the exconjugant cultures (Oa, Ob, Oc, and Od) as compared with that of the parent culture (Fa).

2. The division rate is also markedly higher than that of Fc, a sister culture of Fa, which survived Fa for twelve periods and attained 293 generations.

3. The exconjugant cultures were all dividing rapidly when the parent culture died. However, since the death of the latter can hardly be considered 'normal,' no conclusion regarding relative vitality is justified.

13. Group T. Cultures Ta, Tb, Tc, and Td were started with exconjugants, on March 30, 1921, from two pairs of conjugants taken from Fa on March 29, 1921, the day the latter died (as already described). The results from the T group, constituting another representative of the F_4 generation through cultures Aa and Fa, as shown in figure 15 are similar to those from the O group, viz.:

1. A marked increase in the division rate of all the exconjugant cultures as compared with that of the parent culture (Fa) during the ten periods before its death. The division rate is also markedly higher than that of Fc, a sister culture of Fa, which survived the latter for twelve periods.

2. The parent culture died the day that the conjugants, which gave rise to these cultures, were secured, and therefore conclusions regarding relative vitality are not possible.

14. Group Y. This group was established with two pairs of conjugants from Ta and constitute the F_4 generation through Aa, Fa, and Ta. One culture (Ya) died after four divisions, leaving Yb, Yc, and Yd which were carried for three periods and then stopped. The results plotted in figure 16 show:

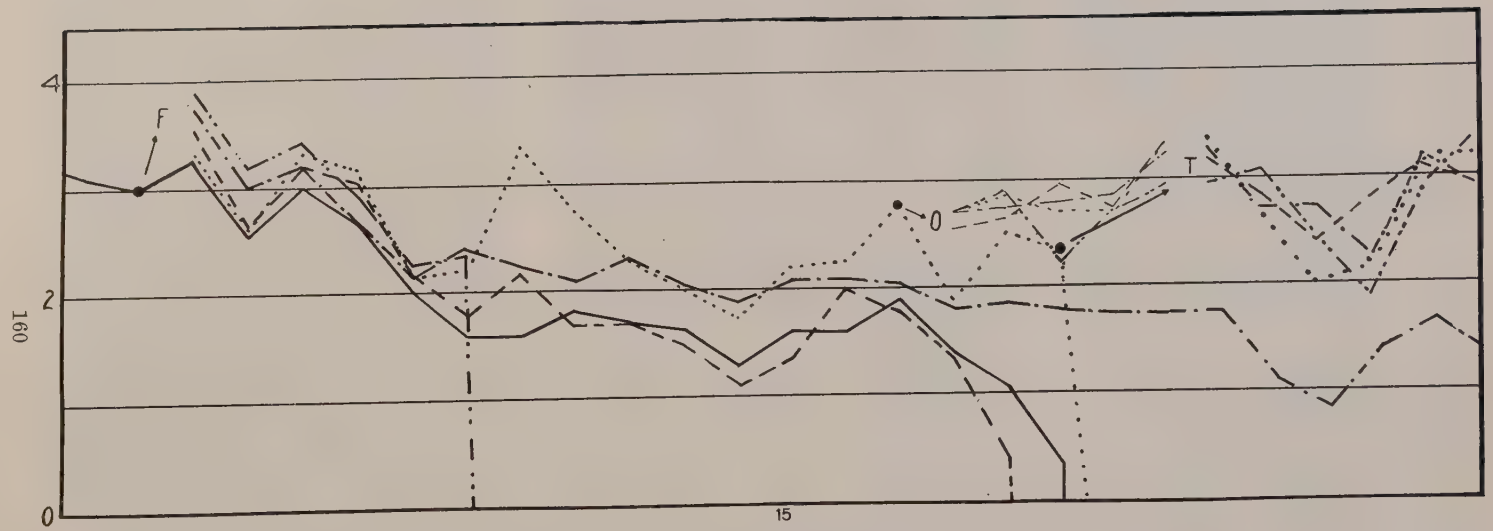


Fig. 15 Graph of the division rate of group F from culture Aa; group O from culture Fa, and group T from culture Fa. (In F group) Aa, — ; Fa, . . .; Fb, – – – ; Fc, –.–; Fd, –. .– ; (in O group) Oa, ...; Ob, – – – ; Oc, –.– ; Od, –. .– ; (in T group) Ta, ...; Tb, – – – ; Tc, –.–; Td, –. .–.

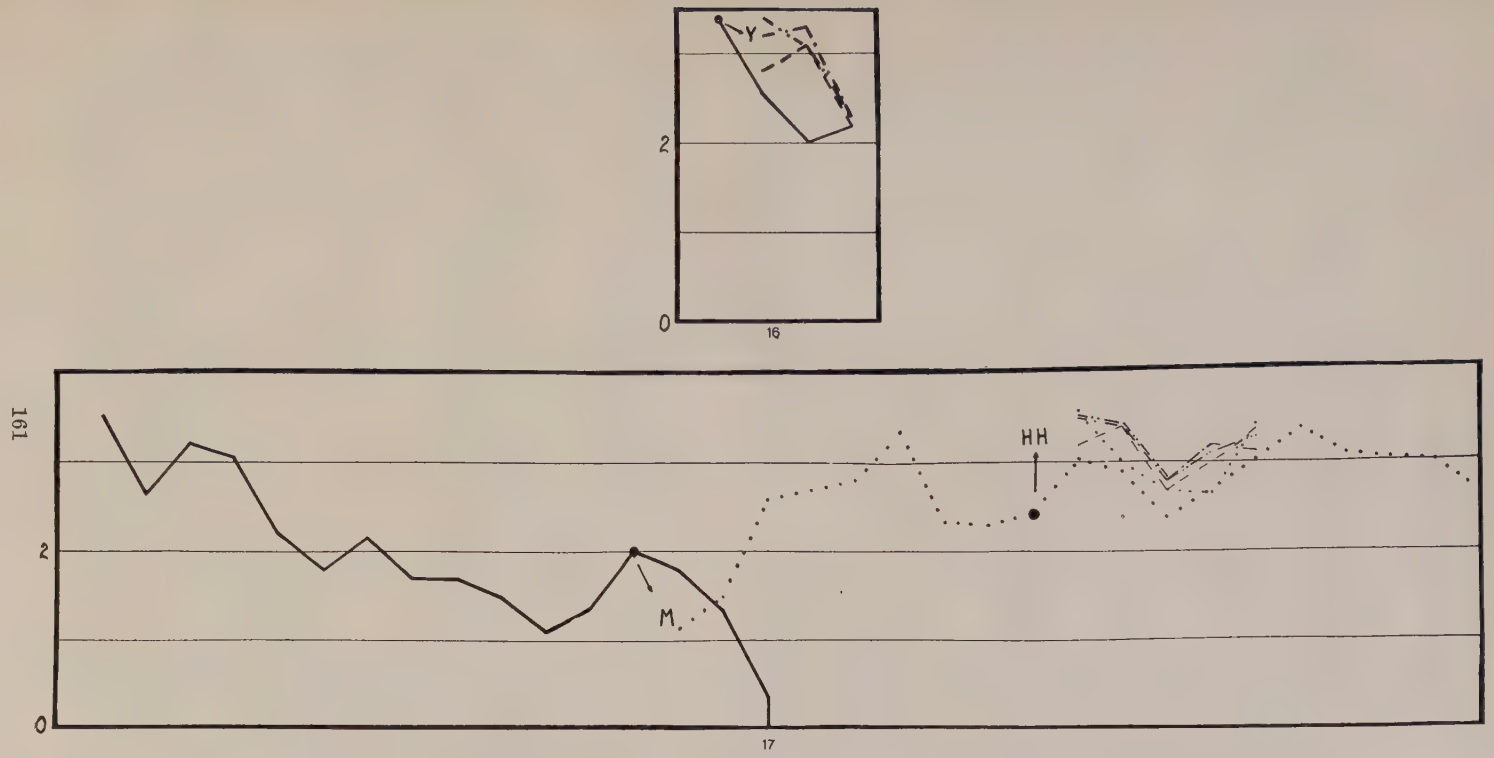


Fig. 16 Graph of the division rate of group Y from culture Ta. Ta, — ; Yb, – – – ; Ye, –.–; Yd, –. .–.

Fig. 17 Graph of the division rate of group M from culture Fb and HH from Ma. Fb, — ; Ma, ... ; HHa, ... ; HHb, – – – ; HHc, –.– ; HHd, –. .–.

An initial marked rise in division rate during the first two periods, and during the final period a decline almost to that of the parent culture (Ta). During the fifteen days of the experiment, the Y group averaged forty-three divisions and Ta showed thirty-four divisions, indicating a significant rise in division rate in the exconjugant cultures.

15. Group M. The Ma and Mb cultures were derived from a pair of conjugants from Fb and represent the F_3 generation. Mb died as an exconjugant without dividing so that the experiment is solely concerned with Ma. Figure 17 shows:

1. The exconjugant culture (Ma) divided less rapidly than the parent culture (Fb) during the first period, but exceeded that of the latter during the remainder of its life. While the two cultures were running synchronously, Ma divided thirty-one times, and Fb, eighteen times; a clear-cut acceleration following conjugation.

2. Ma was stopped at the sixteenth period after the death of Fb, and during the sixteen periods 229 generations were attained, thus showing increased longevity following conjugation.

16. Group HH. HHa, HHb, HHc, and HHd were started with the progeny of two pairs of conjugants from Ma, and represent the F_4 generation through Aa, Fb, and Ma. From figure 17 it is clear that:

Although the HH group arose as exconjugants from Ma when the latter was dividing at the comparatively high rate of two and one-half times a day, nevertheless the HH group divided somewhat more rapidly until it was discontinued after five periods.

17. Group G. We now turn from the groups of cultures from culture Aa through group F, to Group G and its derivatives (table 1 and fig. 7).

Ga, Gb, Gc, and Gd were the progeny of four exconjugants from two pairs of Aa, and therefore represent the F_2 generation from culture I. Ga was discontinued at the end of period 33; and Gc and Gd suddenly died when dividing rapidly at period 9, and Gb at period 16. Figures 18a and 18b show:

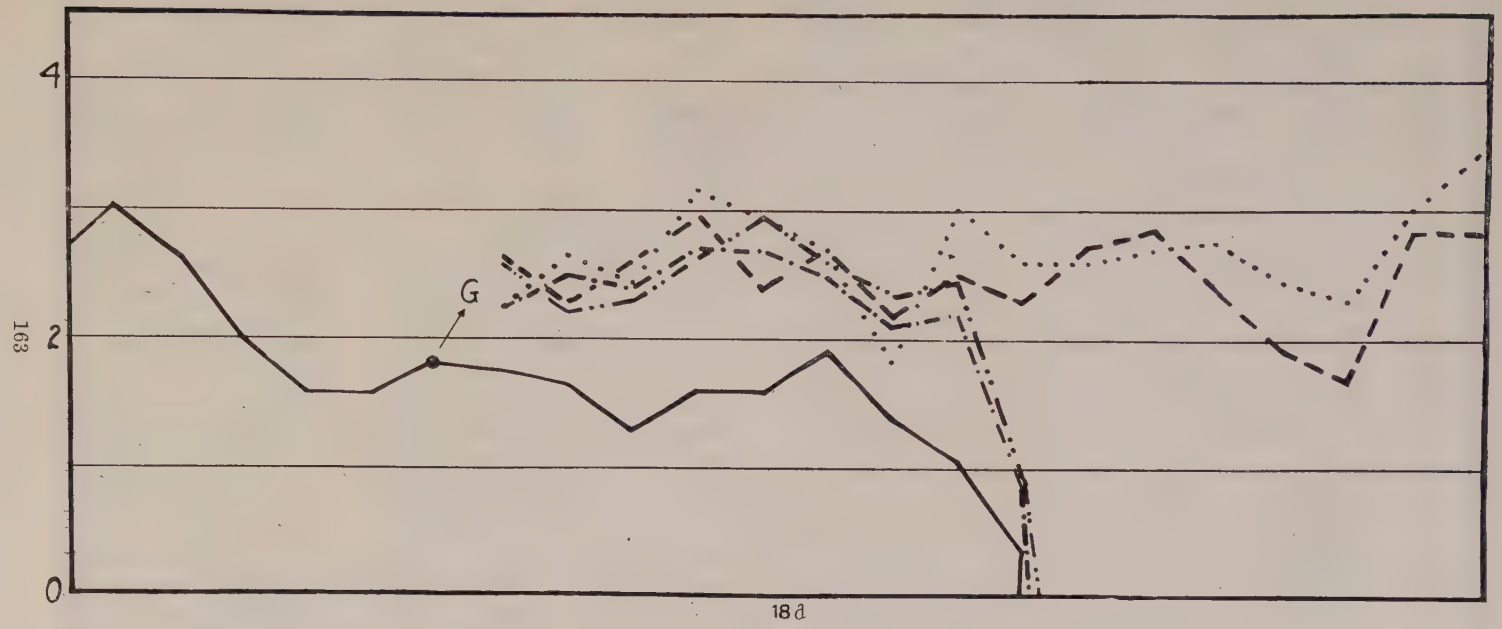


Fig. 18 a Graph of the division rate of group G from culture Aa. Aa, —; Ga, ...; Gb, – – – –; Gc, –.–; Gd, –. .–.

1. An immediate and marked increase in the division rate of all the exconjugant lines in comparison with that of the parent culture (Aa). While the five cultures were running synchronously, Aa divided 67 times, while Ga divided 122 times; Gb, 112 times; Gc, 109 times, and Gd, 108 times.

2. The parent culture died in the ninth period of the experiment after a long decline, and at the same period Gc and Gd suddenly died when dividing rapidly, and Gb did the same at period 16. These sudden deaths are apparently similar to several others already described, and for which no explanation is offered by the vitality as indicated in the division rate or otherwise. Taken at their face value, these data indicate identical longevity of Gc and Gd with that of Aa. On the other hand, Gb when it died had survived Aa by seven periods, and similarly Ga when stopped at period 33 had survived Aa by twenty-four periods, thus indicating a marked increase in longevity as well as a sustained high division rate in these exconjugant cultures.

18. Group K. The progeny of two pairs of conjugants from Ga instituted the K group, representing the F_3 generation through Aa and Ga. The data of this experiment, plotted in figure 19, indicate as follows:

1. A striking difference in the division rate of the progeny of one pair of conjugants (Ka and Kb) from that of the progeny of the other pair (Kc and Kd). Kc and Kd showed an initial acceleration of the division rate which lasted for four periods. Then, for unknown cause, Kc suddenly died when averaging two divisions a day, while Kd continued at a rate averaging slightly lower than that of the parent culture (Ga).

2. The first division of each of the exconjugants (Ka and Kb) was unusually long deferred, and the rate of division of the cultures throughout their life was consistently lower than that of the parent culture (Ga) and of the cultures (Kc and Kd) derived from the other pair of conjugants.

3. Ka and Kb divided at a remarkably similar rate and with but slight fluctuations, between one and two divisions

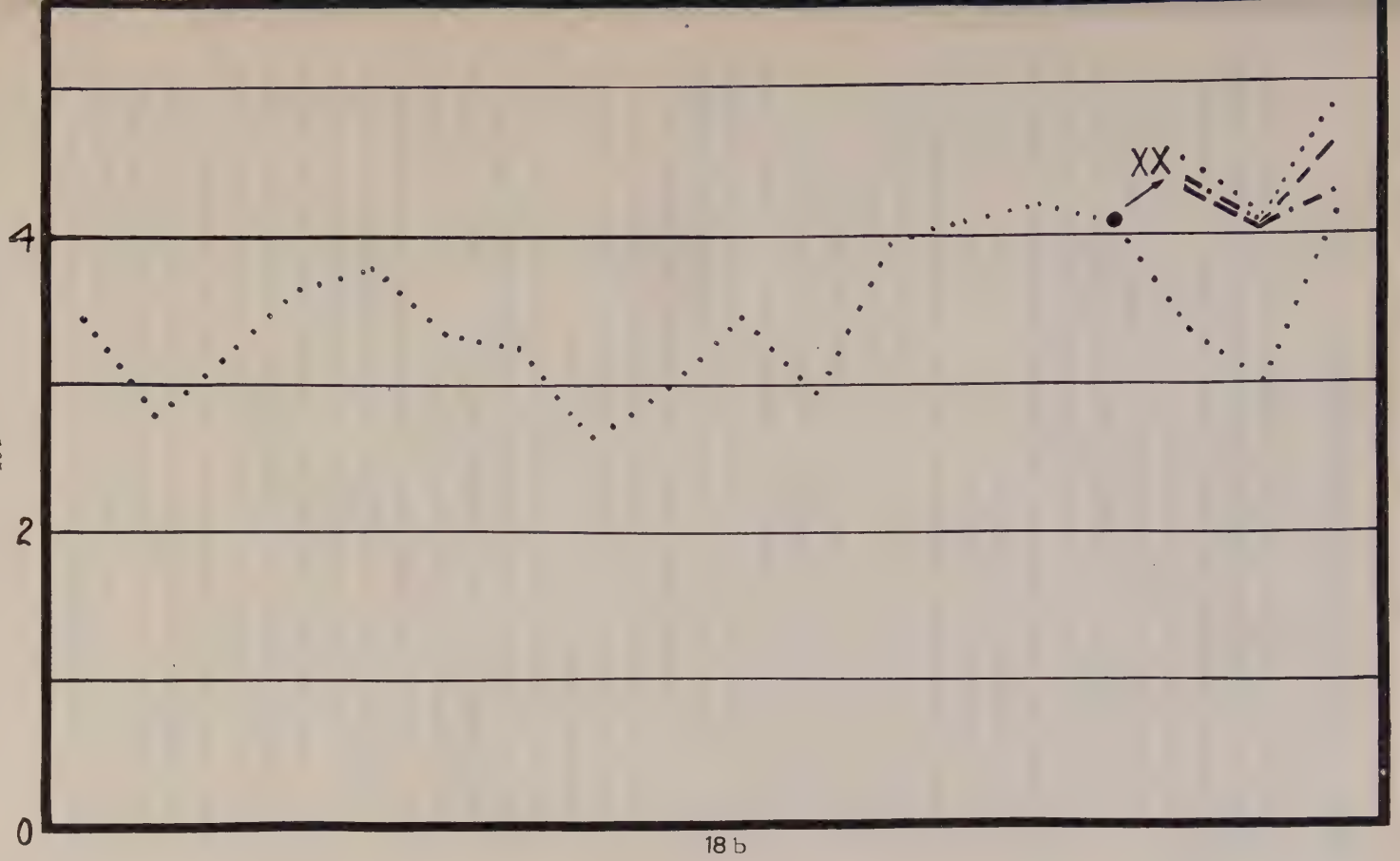


Fig. 18 b Graph of the division rate of group XX from culture Ga. Ga, . . .; XXa, . . .; XXb, – – –; XXc, –.–.

per day, throughout the experiment. No tendency toward loss of vitality and death was shown when they were stopped at the end of thirty periods—Ka at the 210th, Kb at the 235th, and Kd at the 460th generation.

19. Group XX. Two pairs of exconjugants from Ga started cultures XXa, XXb, and XXc. One exconjugant (XXd) died without dividing. The remaining three cultures, which were carried for three periods and then discontinued, gave the following results (cf. fig. 18b):

1. Markedly high division rate in all the exconjugant cultures in comparison with that of the parent cultures; XXa finally reaching an average of nearly five divisions per day—the highest attained throughout all these experiments.

2. All the XX cultures were stopped at the end of three periods, so no data in regard to longevity is available.

20. Group ZZ. A pair of conjugants secured from Kb constituted cultures ZZa and ZZb. ZZa died as an exconjugant, while ZZb continued and afforded the data plotted in figure 19.

1. ZZb divided nearly twice as rapidly as the parent Kb culture throughout the three periods that they were conducted simultaneously; ZZb dividing thirty-two times, and Kb eighteen times.

2. Both cultures were stopped at the same time, and therefore no data in regard to relative viability were secured.

21. Group J. Four cultures (Ja, Jb, Jc, Jd) were started from the exconjugants of two pairs from Aa on February 24, 1921. They represent the F_2 generation from culture I through Aa. The data presented in figure 20 show:

1. All the J cultures divided at a rate about twice that of the parent culture (Aa) during the first three periods and at a consistently higher rate than Aa throughout its life. During these six periods Ja divided 78 times; Jb, 81 times; Jc, 84 times, and Jd, 77 times, in contrast with Aa, 39 times.

2. Three of the exconjugant cultures suddenly died, coincidently with the death of the parent culture, at the end of the sixth period of the experiment when dividing more than twice daily. The significance of this is not apparent, since the

sister culture (Jc) continued with unabated division energy until it was discontinued after 177 generations in period 11. Thus three cultures indicated no change in longevity following conjugation, while one possessed the power to survive the parent non-conjugating culture at least for ninety-three generations, which it had attained when it was stopped.

22. Group N. The progeny of a pair of exconjugants from Ja started cultures Na and Nb, representing the F_3 generation from culture I through Aa and Ja. Figures 21 and 22 show:

1. An increase in the division rate for the three periods during which Na and Nb were running synchronously with Ja.

2. Owing to the sudden extinction of Ja, as already mentioned, it is probably unfair to draw any conclusions in regard to the effect of conjugation on longevity; but, in fact, Na survived Ja for eight periods and Nb survived Ja for eighteen periods when it was discontinued.

23. Groups KK and KK_1. A pair of conjugants from Nb gave rise to cultures KKa and KKb representing the F_4 generation from the stem culture (I) through Aa, Ja, and Nb. The derivatives of both the KK exconjugants showed some conjugating pairs the day following the starting of the cultures. KKa was discarded because it was impossible to make sure that there were any animals which had not 'reconjugated.' In KKb, however, animals, which obviously had not 'reconjugated,' were secured to continue the culture. Figure 21 shows that KKb divided slightly more rapidly than the parent culture (Nb), but the increase is insignificant.

One of the conjugating pairs from KKb was employed to start cultures KK_1a and KK_1b. Thus the KK_1 group was derived from culture I through Aa, Ja, Nb, and KKb. Figure 21 shows that both KK_1 and KK_1b exhibited a marked acceleration of the division rate in comparison with KKb and also with Nb. This was sustained for five periods (three of which are plotted) when they were discontinued.

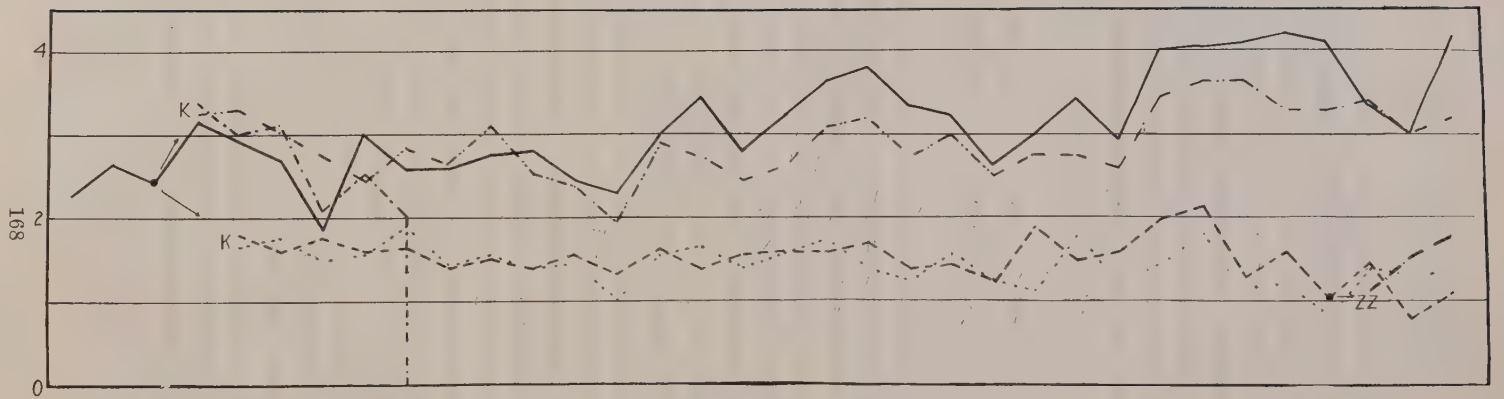


Fig. 19 Graph of the division rate of group K from culture Ga. Ga, — ; Ka, . . . ; Kb, – – – ; Ke, –.– ; Kd, –. .– ; ZZb, –.–.

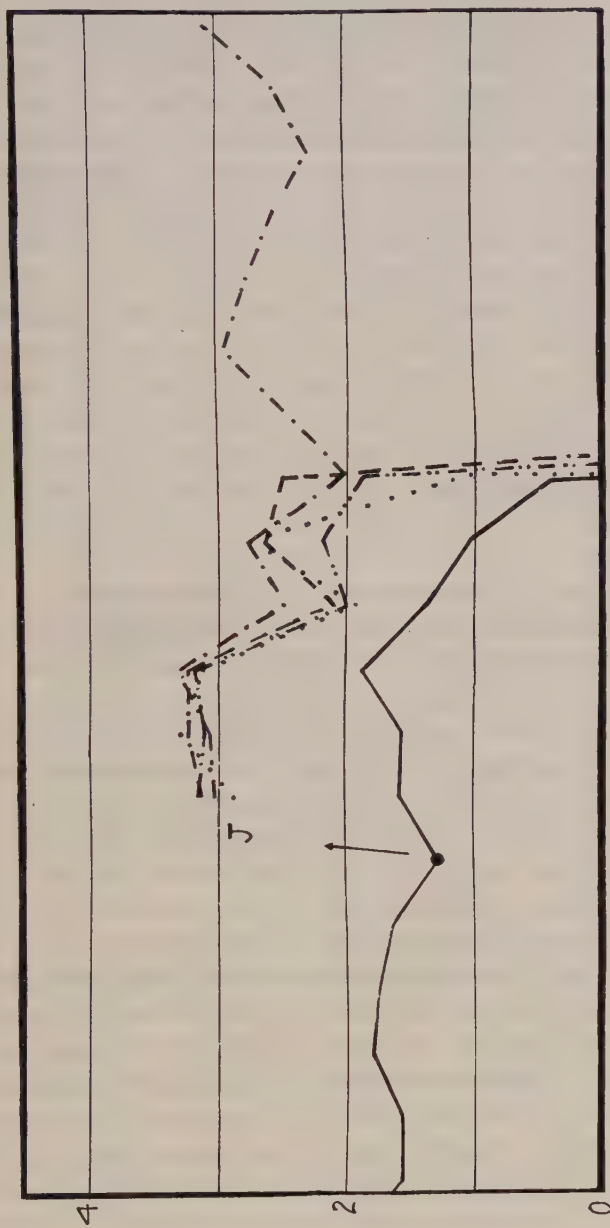


Fig. 20 Graph of the division rate of group J from culture Aa. Aa, —; Ja, . . .; Jb, – – –; Jc, –.–; Jd, –.–.

Throughout these experiments it was found that the descendants of exconjugants usually would not conjugate until about fifteen days had elapsed. Accordingly, the onset of conjugation in the KK group within the first five days after its origin suggests the possibility that fusion had not actually resulted in the usual nuclear reorganization. This suggestion is made still more probable since a significant acceleration in division rate was not exhibited, whereas it was shown by the progeny of the 'reconjugants' (group KK_1). At all events, the KK and KK_1 groups do not present unequivocal answers to any of the questions under investigation, but the data are presented for their intrinsic interest, since they raise the question whether the several cases on record in the literature, which purport to show that Infusoria may successfully 'reconjugate' almost immediately, may not actually be cases where the internal nuclear phenomena were not consummated in the first instance.

24. Group GG. This represents still another group derived from Nb and was started from the exconjugants of two pairs designated GGa, GGb, GGc, and GGd. Figure 22 shows:

All four of the GG cultures exhibited a more rapid division rate than the parent culture (Nb) during the first seven periods. Thereafter, until all the five cultures were stopped, GGa maintained a clear-cut higher rate than Nb, while GGb, GGc, and GGd fell to a level not significantly different from Nb. Throughout the thirteen periods when groups GG and Nb were running synchronously, GGa attained 202; GGb, 190; GGc, 179; GGd, 190, and Nb, 176 generations.

25. Group Q. This group was started with two pairs of conjugants from Ja, five days after the similar origin of the N group. Qa, Qb, and Qc were stopped at the end of five periods where they had attained 72, 76, and 80 generations, respectively. Qd was stopped in the eleventh period at the 168th generation. From figure 23 it is evident that:

1. All four cultures exhibited an initial higher rate of division than the parent culture, Ja.

2. The sudden extinction, already mentioned, of Ja makes it unfair to draw any conclusions in regard to the effect of conjugation on longevity; but, in fact, all the Q cultures continued at a high division rate after the death of Ja, and were finally stopped. (The last six periods of the Qd culture are omitted from figure 23.)

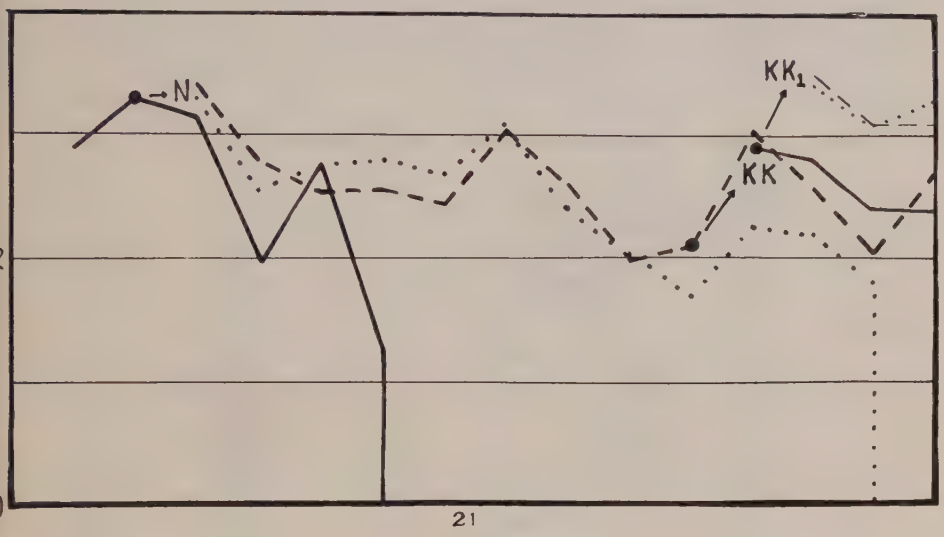


Fig. 21 Graph of the division rate of group N from culture Ja, group KK from culture Nb, and group KK_1 from culture KKb. Ja, — ; Na, ... ; Nb, – – – ; KK, — ; KK_1a, . . . ; KK_1b, – – – .

C. Series E

We turn now to series E, which includes group E, from the stem culture I, and its derivatives. This series duplicates, in general, the experiments constituting series A (fig. 7 and table 1).

26. Group E. Ea and Eb were started with the animals of a pair of conjugants secured from the stem culture I on December 17, 1920, and thus bear the same relation to the parental stem culture as the A group, representing the F_1 generation. The data from the E cultures, plotted in figure 24, show:

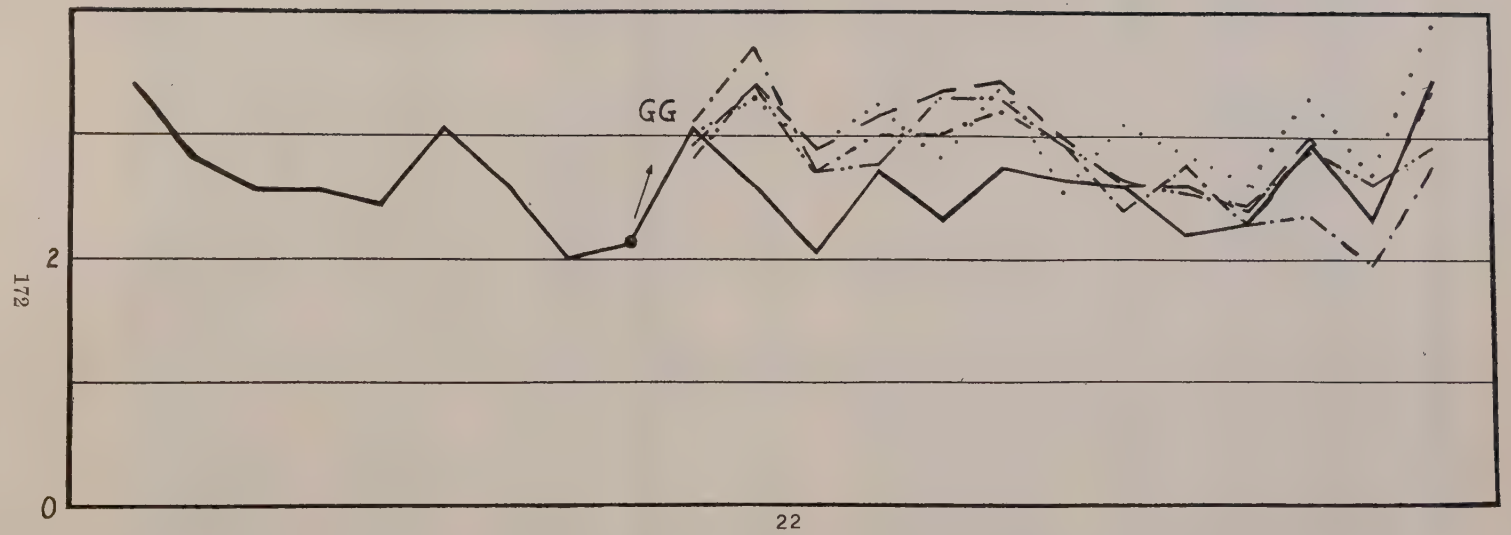


Fig. 22 Graph of the division rate of group GG from culture Nb. Nb, — ; GGa, . . . ; GGb, – – – ; GGc, –.– ; GGd, –. .–.

1. During the first three periods the average division rate of Ea and Eb was significantly lower than the parent culture, and thus affords one of the few clear-cut examples of an initial depression following conjugation.

2. From the fourth period to the twelfth the division rate of both of the E cultures was significantly higher than the parent culture which at this time died after a gradual decline in reproductive activity. Thus the initial low rate of the E cultures was compensated for during the synchronous life of culture I.

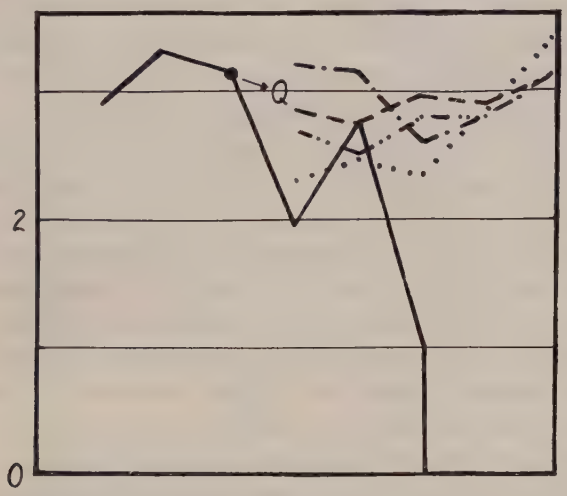


Fig. 23 Graph of the division rate of group Q from culture Ja. Ja, — ; Qa, ... ; Qb, – – – ; Qc, –.– ; Qd, –. .–.

3. When the Ea culture was discontinued at the 211th generation after 125 days in culture, it had survived the parent culture (I) for fourteen periods, and likewise when the Eb culture died at the 267th generation after 145 days in culture, it had survived the parent culture for seventeen periods. Thus the E group affords a clear-cut case of increased longevity following conjugation.

27. Group X. One member of a pair of conjugants from Ea established the Xa culture. The other exconjugant (Xb) was accidentally lost.

The history of Xa from start through five periods, when it was stopped at the 71st generation because of some evidence suggesting that conjugation had occurred between sister cells, is plotted in figure 24, which gives the following results:

1. Very marked initial acceleration of the rate of division following conjugation.

2. Exconjugant culture (Xa) survived the parent culture (Ea), and was dividing over three times per day when it was discontinued, thus indicating an increase of longevity following conjugation.

28. Group CC. Two pairs of conjugants from Xa established cultures CCa, CCb, CCc, and CCd representing the F_3 generation from the stem culture I. The first two immediately encysted, since food, through an accident, was not supplied. CCc and CCd were discontinued at the eighteenth period after attaining 305 and 311 generations, respectively.

Since the parent culture (Xa) had to be stopped on account of the possibility of conjugation in its direct lines, there is not a complete control for CCc and CCd; and therefore no conclusions can be drawn from their record, and a graph is not given. It is mentioned for completeness, and because it was the parent culture of cultures employed in an experiment to be described later (cf. p. 195).

29. Group H. A pair of conjugants were secured from Eb, one of which died immediately and the other constituted the Ha culture. This was bred for eight periods when it died at the 107th generation. Figure 24 shows:

1. A very much higher division rate for Ha than for the parent Eb culture throughout their synchronous life.

2. Ha died suddenly for no apparent cause when dividing rapidly during period 8 of the experiment, while Eb continued normally. The facts as they stand must be interpreted as indicating decreased longevity for the culture which has more recently conjugated.

30. Group S. Two pairs of conjugants from Eb gave four exconjugants, Sa, Sb, Sc, and Sd. Sb and Sc died without dividing. Sa lived for six periods and Sd for two periods. The data for Sa and Sd (not plotted) indicate:

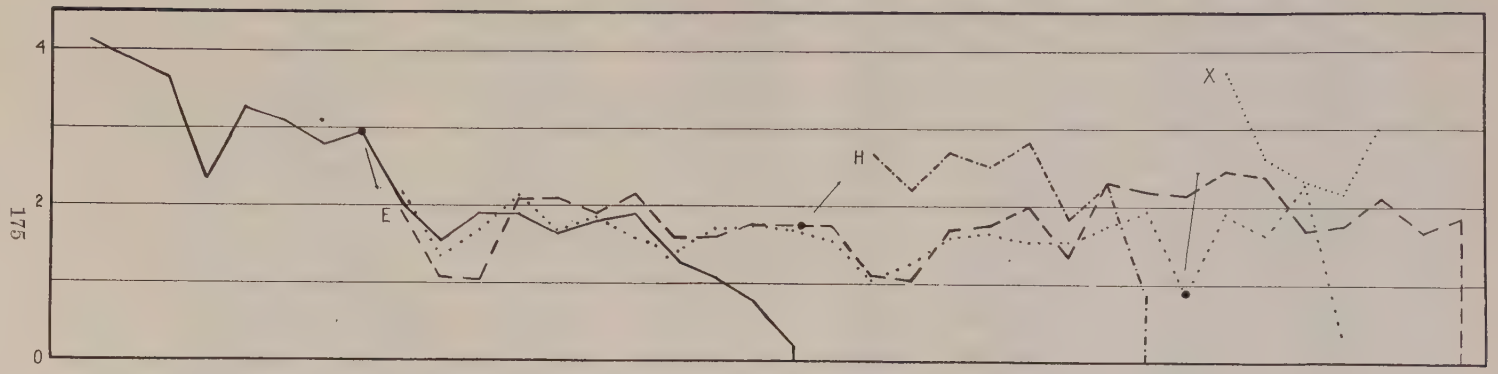


Fig. 24 Graph of the division rate of group E from culture I, group H from culture Eb, and group X from culture Ea.
I, —; Ea, ...; Eb, – – –; Ha, –·–; Xa, ...

1. Sa divided less rapidly than the parent culture and affords a clear-cut case of decreased reproductive activity following conjugation. Sd, on the other hand, during the two periods it lived, divided markedly more rapidly than the parent culture and so gives evidence opposite to that from Sa.

2. Both Sa and Sd died while Eb was still dividing rapidly, so both the S cultures indicate a decrease in longevity following conjugation.

31. Group DD. Two pairs of conjugants from Eb started the DD cultures. One exconjugant from each pair died without dividing, leaving DDa and DDd for study. These were stopped at the end of the third period. From figure 25 it is clear that:

1. DDa and DDd divided more rapidly than the parent Eb culture—a clear-cut instance of accelerated reproductive rate following conjugation.

2. No data regarding relative longevity are available.

32. Group LL. LLa, LLb, LLc, and LLd represent the progeny of two pairs of conjugants from Eb. All four cultures were discontinued at the end of the tenth period. The data for the first three periods are plotted in figure 25, which shows:

1. A very much higher division rate in all the LL cultures than in the parent culture (Eb), thus affording a clear-cut case of acceleration of reproduction following conjugation during the same periods. As a matter of fact, the division rate of all the LL cultures was higher than the parent culture ever attained (cf. fig. 24).

2. All four cultures survived the parent culture for eight periods (later periods omitted in fig. 25), and then were discontinued when dividing over three and one-half times per day. Group LL thus affords a pronounced example of increased longevity following conjugation.

33. Group NN. The direct lines of the Eb culture died on May 9, 1921, but from 'stock' remaining from the isolations of two days before, three conjugating pairs were taken and, on May 11th, as exconjugants established the NN group. One

exconjugant from each pair died without dividing, leaving cultures NNa, NNc, and NNe for study. The data derived from the NN group during the three periods it was carried are shown in figure 25, from which it is apparent that:

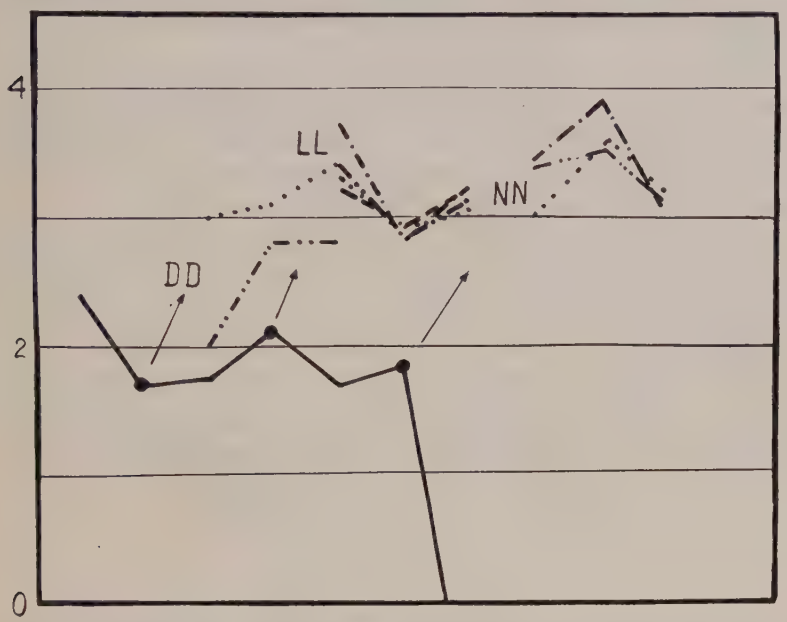


Fig. 25 Graph of the division rate of group DD from culture Eb, group LL from culture Eb, and group NN from culture Eb. Eb, — ; DDa, ... ; DDd, –.– ; LLa, . . . ; LLb, – – – ; LLc, –.–; LLd, –. .–; NNa, ... ; NNc, –.– ; NNe, –. . . –.

1. All NN cultures had a markedly higher division rate than the parent Eb culture ever exhibited (cf. fig. 24). Thus these cultures afford a striking example of accelerated reproduction following conjugation.

2. The parent culture died before the NN cultures were established as exconjugants; therefore the latter afford a striking instance of the survival of exconjugants after the death of the 'non-conjugant' parent.

RESULTS AND DISCUSSION

With the data now presented in their entirety, it is advisable to restate and summarize the evidence afforded toward a solution of the basic problem—the effect of conjugation on the reproductive activity of this pedigree race of Spathidium spathula under laboratory conditions.

This may be appropriately approached by considering first, whether Spathidium can continue indefinitely to reproduce by division without the periodical advent of endomixis or conjugation. This answered, either positively or negatively, paves the way for the following questions:

Do the progeny of exconjugants characteristically exhibit a significantly different (higher or lower) rate of reproduction from that of their parent culture which has not so recently conjugated?

Do the progeny of exconjugants characteristically exhibit an increase or decrease in longevity in comparison with their parent culture which has not so recently conjugated?

These questions will be taken up seriatim.

A. The 'life-cycle'

Can Spathidium reproduce indefinitely without endomixis or conjugation? Cytological studies of a large series of animals preserved throughout these experiments have shown, as already stated, that endomixis does not occur in this organism during its active life; while Miss Moore, studying animals from this pedigree race, has found endomixis during encystment. Conjugation has not taken place in the direct lines of the cultures owing to the daily isolation of animals from each pedigree line. Accordingly, neither conjugation nor endomixis has occurred during the life of the direct pedigree lines during these experiments under consideration.

The data presented show that in the great majority of cases the cultures died out sooner or later after a somewhat gradual decline in division rate. But there is no evidence of a 'cycle' definite in regard to duration of time or number of genera-

tions. Thus, for example, the stem culture (I) lived 95 days and attained 234 generations; Aa, 130 days and 338 generations (fig. 8 and table 1); Da, 125 days and 272 generations (fig. 9), and Fb, 80 days and 169 generations (fig. 15). In many cultures no marked decline occurred and the cultures were stopped when the definitive experiments were concluded. For example, Ga had already lived 165 days and attained 546 generations (figs. 18a and 18b); Ka, 150 days and 210 generations; Kb, 150 days and 234 generations, and Kd, 150 days and 460 generations when purposely stopped (fig. 19).

In view of the positive evidence afforded by the long life of certain cultures such as those just mentioned, and especially by another culture now under investigation definitely in regard to this point for over six months, which has already attained nearly 600 generations without evidence of waning vitality in the absence of conjugation and endomixis, the relatively brief life of the majority of cultures described in this paper cannot be interpreted as evidence of an inherent tendency to senile degeneration in the Maupasian sense.

The entire trend of research in recent years has definitely indicated that limited 'cycles' are the result of unfavorable culture conditions. Specifically, this has been shown to be the case by Woodruff on Paramecium aurelia and Paramecium caudatum, Enriques on Glaucoma scintillans, Stylonychia pustulata, and Vorticella nebulifera, Baitsell on Pleurotricha lanceolata and Oxytricha fallax, and Dawson on Oxytricha hymenostoma. Hartmann's recent work on Eudorina elegans points in the same direction. In a word, all these studies[18] indicate that conjugation is not necessary for continued life and reproduction.

But the discovery of a nuclear reorganization process in Paramecium aurelia and Paramecium caudatum offered another complication, since it was found that the cultures died out when endomixis was suppressed. Although this is certainly true for the races of P. aurelia and P. caudatum which

[18] References previously cited.

were studied, a race of Paramecium calkinsi[19] which has been bred in this laboratory for more than three years without conjugation has, during this period, as demonstrated by Spencer, never undergone endomixis. Thus it is clear that Paramecia, in which endomixis is established as a periodic phenomenon, are unable to survive its suppression, while others live indefinitely without endomixis as well as conjugation. Further, there is no evidence that endomixis occurs in the free-living stages of any of the other species of ciliates which have been studied from the point of view under discussion. Accordingly, a wealth of evidence points definitely to the conclusion that neither endomixis nor conjugation is indispensable and that reproduction by division may, in general, continue indefinitely in their absence.

Of especial importance in regard to this problem is the recently published series of studies by Calkins on Uroleptus mobilis. Calkins clearly shows that under the conditions of pedigree culture this organism gradually undergoes a waning of vitality which invariably terminates in the death of the strain unless conjugation occurs.

But we are unable to grant that the decrease in vitality which he found in Uroleptus is due to an intrinsic tendency to 'senility' in the absence of conjugation or endomixis. As already stated, the crucial factors of the milieu to which certain species of Infusoria are adapted are too subtle to allow the assumption that essentially ideal ones can be readily provided, and this is particularly true in the case of Uroleptus mobilis. The extreme rarity of this species may reasonably be attributed to peculiar conditions of life demanded, and in view of the wealth of positive evidence cited above attesting that, when suitable conditions are provided, conjugation and endomixis (except in P. aurelia and P. caudatum) are not indispensable, the negative evidence from Uroleptus is more reasonably regarded as due to environmental factors. And we reach the same conclusion in regard to Spathidium. It is

[19] Woodruff: The structure, life history, and intrageneric relationships of Paramecium calkinsi, sp. nov. Biological Bulletin, vol. 41, 1921.

clear that many of the cultures of Spathidium employed in these experiments have gradually declined in vitality and died in the absence of conjugation and endomixis, but we believe that this result must be interpreted as arising from conditions which are extrinsic and not fundamental.[20]

B. The effects of conjugation on the division rate

1. General data. A study of tables 1 and 2 shows that a total of 118 exconjugants contributed to the experimental results. Of these, in two cases (C, R) both exconjugants from a pair died without dividing; in fourteen cases (Hb, Mb, Sb, Sc, DDb, DDc, NNb, NNd, NNf, XXd, YYb, YYd, YYf, ZZa) one exconjugant of a pair died without dividing. That is, eighteen exconjugants among the 118 involved in the definitive experiments gave a positive result—death immediately followed conjugation.

However, the proportion of deaths is so small that it is negligible from the standpoint of this study. It is lower than the exigencies involved in such experimental work would naturally supply when the isolations and manipulations necessary are considered. Indeed, as already stated (p. 139), the high viability of exconjugants in this race of Spathidium is remarkable in comparison with the results obtained by most investigators studying conjugation in other Infusoria. Accordingly, we shall omit these eighteen cases from further consideration, though the reader, of course, may weigh them as he sees fit in judging the validity of our conclusions. In addition to the cases just considered, six other exconjugants (Sd, Xb, Ya, CCa, CCb, and KKa) need not be considered for obvious reasons indicated in table 1. Thus there remain ninety-four exconjugants which established cultures affording cogent data already presented.

Seventy-nine of these ran synchronously with their parental cultures for three or more periods, and so afford definite evidence bearing on the question: Do the progeny of

[20] Woodruff and Moore: On the longevity of Spathidium spathula without endomixis or conjugation, Proc. Nat. Acad. Sci., 1924. (In press.)

TABLE 2

CULTURE	AVERAGE DIVISION RATE FOR 15 DAYS	AVERAGE NUMBER OF DIVISIONS ABOVE OR BELOW PARENT CULTURE FOR 15 DAYS	PERIODS EXCONJUGANT EXCEEDED PARENT[1]	FATE: DIED OR STOPPED	NUMBER OF DIVISIONS EXCONJUGANT EXCEEDED PARENT[1]	NUMBER OF DIVISIONS EXCONJUGANT EXCEEDED PARENT DURING CONTEMPORARY PERIODS[2]	NUMBER OF CONTEMPORARY PERIODS	TEXT FIGURE NUMBER
I	46.3							
Aa	56.5	10.2	9	d	63	71	17	8
Ab	54.3	8.0	7	d	32	32	14	
Ac	50.8	4.5	11	d	130	13	17	
Ad	49.8	3.5	4	d	14	38	17	
I	32.8							
Aa	51.5							
Da	44.3	−7.2	4	d	46	35	22	9
Db	46.0	−5.5	6	d	65	38	22	
Aa	24.5							
Da	34.8							
La	44.5	9.7	1	s	22	64	9	10
Lb	40.8	3.7	−3	d	—	17	6	
Lc	41.5	3.0	−3	d	—	17	6	
Ld	34.5	−0.3	−3	d	—	16	6	
Aa	14.0							
Da	24.0							
Lc	32.8							
Pa	32.3	−0.5	2	s	32	2	3	11
Pb	31.8	−1.0	2	s	33	3	3	
Da	3.3	(died within 15 days)						
Ld	dead							
Ua	42.8	—	3	s	49	—	—	12
Ub	40.0	—	3	s	50	—	—	
Uc	40.5	—	3	s	49	—	—	
Ud	44.3	—	3	s	53	—	—	
Db	16.3							
Wa	44.5	28.2	−1	d	—	24	3	13
Wb	41.3	25.0	−1	d	—	25	3	
Wc	36.3	20.0	7	s	85	20	5	
Wd	42.8	26.5	7	s	66	25	5	

[1] In the interpretation of these data, the fate (died or stopped) obviously must be considered. Since cultures were not stopped when showing any indications of impending death, it is clear that the longevity of stopped cultures is in all cases potentially in excess of the figures given.

[2] These data obviously must be interpreted in conjunction with those in the following column.

TABLE 2—*Continued*

CULTURE	AVERAGE DIVISION RATE FOR 15 DAYS	AVERAGE NUMBER OF DIVISIONS ABOVE OR BELOW PARENT CULTURE FOR 15 DAYS	PERIODS EXCONJUGANT EXCEEDED PARENT[1]	FATE: DIED OR STOPPED	NUMBER OF DIVISIONS EXCONJUGANT EXCEEDED PARENT[1]	NUMBER OF DIVISIONS EXCONJUGANT EXCEEDED PARENT DURING CONTEMPORARY PERIODS[2]	NUMBER OF CONTEMPORARY PERIODS	TEXT FIGURE NUMBER
Db	dead							
EEa	32.0	——	4	d	51	—	—	14
EEb	33.5	——	17	d	158	—	—	
EEb	20.0							
TTa	54.0	34.0	3	s	50	28	5	14
TTb	54.5	34.5	3	s	56	34	5	
TTc	44.8	24.8	3	s	47	25	5	
TTd	51.5	31.5	3	s	57	35	5	
TTa	54.8							
YYa	63.0	8.2	19	s	241	10	3	14
YYc	61.8	7.0	—	s	—	9	3	
YYe	64.0	9.2	—	s	—	10	3	
I	25.3							
Aa	44.0							
Fa	46.0	2.0	—	d	—	63	17	15
Fb	47.0	3.0	–1	d	—	4	16	
Fc	49.8	5.8	12	d	90	51	17	
Fd	52.5	8.5	–11	d	—	17	6	
Aa	14.0							
Fa	33.8							
Oa	41.3	7.5	2	s	31	6	3	15
Ob	40.8	7.0	2	s	29	6	3	
Oc	41.3	7.5	2	s	29	9	3	
Od	39.3	5.5	2	s	34	6	3	
Fa	dead							
Ta	40.3	——	6	s	91	—	—	15
Tb	42.3	——	6	s	96	—	—	
Tc	43.5	——	6	s	94	—	—	
Td	43.8	——	6	s	91	—	—	
Ta	34.3							
Yb	40.5	6.2	—	s	—	2	5	16
Yc	44.0	9.7	—	s	—	6	5	
Yd	44.0	9.7	—	s	—	7	5	
Aa	21.8							
Fb	17.8							
Ma	26.3	8.5	15	s	216	9	5	17

TABLE 2—*Continued*

CULTURE	AVERAGE DIVISION RATE FOR 15 DAYS	AVERAGE NUMBER (+) OF DIVISIONS ABOVE OR BELOW PARENT CULTURE FOR 15 DAYS	PERIODS EXCONJUGANT EXCEEDED PARENT[1]	FATE: DIED OR STOPPED	NUMBER OF DIVISIONS EXCONJUGANT EXCEEDED PARENT[1]	NUMBER OF DIVISIONS EXCONJUGANT EXCEEDED PARENT DURING CONTEMPORARY PERIODS[2]	NUMBER OF CONTEMPORARY PERIODS	TEXT FIGURE NUMBER
Ma	41.8							
HHa	46.5	4.7	–5	s	—	3	5	17
HHb	46.5	4.7	–5	s	—	7	5	
HHc	48.5	6.7	–5	s	—	8	5	
HHd	48.5	6.7	–5	s	—	8	5	
Aa	23.3							
Ga	36.8	13.5	24	s	421	58	9	18 a
Gb	37.8	14.5	7	d	92	46	9	and
Gc	35.8	12.5	—	d	—	42	9	18 b
Gd	35.8	12.5	—	d	—	41	9	
Aa	24.5							
Ga	37.5							
Ka	24.5	–13.0	—	s	—	–287	30	19
Kb	25.8	–11.7	—	s	—	–262	30	
Kc	41.0	3.5	–25	d	—	–1	5	
Kd	45.5	8.0	—	s	—	–37	30	
Kb	16.8							
ZZb	29.3	12.5	—	s	—	11	3	19
Ga	52.5							
XXa	67.3	14.8	—	s	—	23	3	18 b
XXb	69.8	17.3	—	s	—	22	3	
XXc	63.5	11.0	—	s	—	18	3	
Aa	25.5							
Ja	46.8	21.3	—	d	—	37	6	20
Jb	48.3	22.8	—	d	—	42	6	
Jc	47.3	21.8	7	s	91	47	6	
Jd	47.3	21.8	—	d	—	37	6	
Aa	21.8							
Ja	39.3							
Na	43.0	3.7	8	d	90	31	4	21 and
Nb	43.8	4.5	18	s	240	29	4	22
Nb	38.5							
KKb	40.5	2.0	–8	s	—	5	5	21

TABLE 2—*Continued*

CULTURE	AVERAGE DIVISION RATE FOR 15 DAYS	AVERAGE NUMBER OF DIVISIONS ABOVE OR BELOW PARENT CULTURE FOR 15 DAYS	PERIODS EXCONJUGANT EXCEEDED PARENT[1]	FATE: DIED OR STOPPED	NUMBER OF DIVISIONS EXCONJUGANT EXCEEDED PARENT[1]	NUMBER OF DIVISIONS EXCONJUGANT EXCEEDED PARENT DURING CONTEMPORARY PERIODS[2]	NUMBER OF CONTEMPORARY PERIODS	TEXT FIGURE NUMBER
Nb	36.8							
KKb	38.0					-		
KK,a	49.0	11.0	—	s	—	13	4	21
KK,b	48.5	9.5	—	s	—	12	4	
Nb	38.5							
GGa	46.0	7.5	—	s	—	26	13	22
GGb	46.0	7.5	—	s	—	14	13	
GGc	47.5	9.0	—	s	—	3	13	
GGd	44.5	6.0	—	s	—	14	13	
Aa	14.0							
Ja	28.8							
Qa	35.5	6.7	3	s	77	8	3	23
Qb	42.8	14.0	3	s	50	14	3	
Qc	44.8	16.0	3	s	51	16	3	
Qd	40.0	11.2	6	s	49	15	3	
I	27.5							
Ea	26.3	−1.2	14	s	120	11	11	24
Eb	20.5	−7.0	17	d	170	12	11	
Ea	29.3							
Xa	43.8	14.5	1	s	23	14	3	24
Xa	(2 periods only)							
CCc	44.0	—	16	s	266	7	2	(not
CCd	46.0	—	16	s	271	8	2	figured)
Eb	19.3							
Ha	38.3	19.0	−8	d	—	27	8	24
Eb	34.0							
Sa	24.0	−10.0	−4	d	—	−26	6	(not figured)
Eb	9.5							
DDa	47.5	38.0	—	s	—	23	4	25
DDd	38.0	28.5	2	s	28	14	4	

TABLE 2—*Continued*

CULTURE	AVERAGE DIVISION RATE FOR 15 DAYS	AVERAGE NUMBER OF DIVISIONS ABOVE OR BELOW PARENT CULTURE FOR 15 DAYS	PERIODS EXCONJUGANT EXCEEDED PARENT[1]	FATE: DIED OR STOPPED	NUMBER OF DIVISIONS EXCONJUGANT EXCEEDED PARENT[1]	NUMBER OF DIVISIONS EXCONJUGANT EXCEEDED PARENT DURING CONTEMPORARY PERIODS[2]	NUMBER OF CONTEMPORARY PERIODS	TEXT FIGURE NUMBER
Eb	17.8							
LLa	46.0	28.2	8	s	—	29	3	25
LLb	46.5	28.7	8	s	—	30	3	
LLc	48.5	30.7	8	s	—	26	3	
LLd	46.5	28.7	8	s	—	23	3	
Eb	dead							
NNa	49.0	—	3	s	53	—	—	25
NNc	52.3	—	3	s	57	—	—	
NNe	50.8	—	3	s	55	—	—	

exconjugants characteristically exhibit a significantly different (higher or lower) rate of reproduction from that of their parent culture which has not so recently conjugated? Some of these cultures were continued for a long time and even beyond the life of their parent cultures. Others were discontinued at the end of the third period (fifteen days) after the first division following conjugation. The comparative division rates of exconjugant and parent cultures during the initial three periods of all cultures will be first considered; the evidence at this time is particularly cogent, since, obviously, the longer cultures are carried, the more chances there are for accidental extraneous factors, beyond experimental control, to affect the animals and make the results insecure.

2. Initial effect. A comparison may now be made of the division rate during the first three periods of the life of exconjugant cultures with the synchronous division rate of their respective parent cultures.

A study of the initial fifteen days of each of the seventy-nine exconjugant cultures, whose parent culture survived three periods after its origin, shows that sixty-seven divided more

rapidly than their respective parent cultures and twelve less rapidly. The fifteen other cultures, whose parental cultures died within the fifteen days, all actually divided more rapidly than their respective parents, so that they may with justice be added to the regular sixty-seven plus cultures to give eighty-two cultures showing an initial increase in the division rate. However, it is immaterial whether the latter cases are included or not, so far as the conclusion which must be drawn is concerned. Obviously, the data overwhelmingly indicate that the rate of reproduction is accelerated after conjugation.

But the analysis thus far has involved merely contrasting the division rate of exconjugant and parent cultures on the basis of an increase or decrease of division rate, without considering the amount of difference. Clearly, before a conclusion is secure, it is necessary to take into account the chance variation which is inevitable in any experiment in animal breeding such as the present. The usual biometrical methods of analysis are inapplicable here because in each experiment the individual cultures must be treated separately, and judged in relation to the synchronous division rate of their respective parent cultures and also of their grandparental cultures (if still surviving), as well as in relation to other cultures.

But the difficulties are more fancied than real, for by any reasonable criterion the number of cases in which the exconjugants exceeded the parent is far and away beyond the limits of any probable error that statistical methods may formulate. For the sake of tabulation, we have adopted a difference of five divisions in three periods, or an average of one-third of a division per day, as a significant variation. Applying this criterion to the data gives, as table 2 shows, fifty-seven exconjugant cultures exceeding their respective parent cultures; six exconjugant cultures below their parent cultures, and sixteen which are within this limit of error. A similar analysis, in which seven divisions in three periods is taken as the significant variation gives fifty plus exconjugants, five minus exconjugants, and twenty-four neutral. But our experience

in handling the hundreds of pedigree lines during this experiment has convinced us that seven divisions in three periods is without doubt unreasonably large, and this is obvious when it is realized that this represents a change in the average rate of nearly one-half a division per day. Nevertheless, even with this ultraconservative standard, the data give an unequivocal answer: conjugation is followed by an acceleration of the division rate, and since recent conjugation is the sole variable involved in exconjugant and parent cultures, it is evident that conjugation directly induces an immediate acceleration of the reproductive activity.

3. Effect in slowly and rapidly dividing cultures. Do exconjugant cultures which are derived from parent cultures dividing slowly show a relatively greater increase in the division rate, during the first three periods (fifteen days) as compared with the parent cultures, than do exconjugant cultures which are derived from parent cultures dividing rapidly? The number of cultures which afford data on this point is, from the nature of the question, somewhat more limited; but there are a few which supply a fairly definite answer. Comparison of the increase in division rate during the initial three periods of groups A (fig. 8), D (fig. 9), and F (fig. 15) with that of groups W (fig. 13), TT (fig. 14), J (fig. 20), H (fig. 24), X (fig. 24), LL (fig. 25), and DD (fig. 25), fairly establishes, we believe, for this pedigree race of Spathidium that conjugation usually is even more effective in accelerating reproduction when the rate of reproduction is low.

4. Later effect of conjugation. It has been shown that conjugation induces an accelerated rate of division during the first fifteen days following, and we turn now to the evidence available in regard to the comparative reproductive rate of exconjugant animals and their parental lines throughout their entire synchronous life.

There are eighty-one cultures which afford available data for the question in hand; two more than for our analysis of the three initial periods, since the parent culture of CCc and CCd was stopped during their second period.

From an examination of table 2, supplemented by the various graphs, it is evident that seventy-six exconjugant cultures exceeded their parental cultures in the total number of divisions attained during their synchronous life and five exconjugant cultures underwent a smaller number of divisions. Obviously, the data indicate overwhelmingly that, following conjugation, the rate of division is accelerated.

But again, this analysis does not take into consideration the amount of difference in division rate between exconjugant and parental lines, and the difficulties involved here in determining a variation which is significant are still greater than in the comparison, just concluded, for the rates during a definite period of fifteen days. For now we are dealing with differences in duration, e.g., some cultures run synchronously for three periods and others for more than a dozen periods. Probably we cannot do better than to take again the average variation of one-third of a division per day, employed in our previous analysis, as the criterion of a significant variation. Certainly this is extremely conservative. Judged from this standard, sixty exconjugant cultures exceeded their parental cultures in division rate during their entire synchronous life; three exconjugant cultures were lower in division rate, and eighteen cultures were within the limit of error of our standard.

Since conjugation is the sole variable involved in exconjugant and parental cultures, it is evident that conjugation directly induces not only an immediate acceleration of reproduction, but also an acceleration which persists at least as long as the life of the parental culture. And it is significant that the data from the first three periods and also from the entire synchronous life are practically identical, since this shows that the initial acceleration is maintained in essentially equal intensity to the end. In brief, these results are in accord with Calkins' results on Uroleptus, and in opposition to all results which indicate that conjugation is devoid of a profound physiological stimulation of the metabolic activities of the cell expressed in reproduction.

C. The effect of conjugation on longevity

1. General survival value. The question now to be considered is: Do the progeny of exconjugants characteristically exhibit an increase or decrease in longevity in comparison with their parent culture which has not so recently conjugated? The various graphs and tables show that seventy cultures afford cogent data. Of these, fifty-four exconjugant cultures continued to live and reproduce after the death of their respective parent cultures, ten exconjugant cultures died before their respective parent cultures, and six exconjugant cultures died essentially synchronously with their respective parent cultures. This analysis includes the cases where the exconjugant cultures when dividing rapidly suddenly died from no apparent intrinsic circumstances, since no criterion exists for positively determining that such deaths were 'accidental.' But since this fate befell more exconjugant than parent cultures, if these were omitted from the analysis the relative number of exconjugant cultures which survived their parent cultures obviously would be increased.

Thus the data presented provide a very definite answer to the question under immediate discussion. In the great majority of cases the exconjugant cultures outlived their parent cultures, and since the advent of conjugation is the sole variable involved, it is apparent that conjugation tends to increase the time duration of life as well as the rate of reproduction in Spathidium. Therefore, necessarily the number of generations attained by the exconjugant culture exceeds that of the parent after the origin of the former. In brief, under the conditions of these experiments, conjugation has a survival value in the life-history of this organism.

The data have now been presented and summarized which directly contribute to the main questions we started out to answer, viz.: Does conjugation significantly alter the division rate and longevity? Our answers on the basis of this study of pedigree Spathidium are definite. Conjugation in the great majority of cases effects significant changes in both: conjugation increases division rate and longevity.

2. Immediate survival value. This conclusion naturally leads to a consideration of the effect of conjugation on animals from cultures which are apparently on the verge of immediate extinction. In short, has conjugation a direct and immediate survival value?

A study of the graphs and tables shows four groups of cultures (T, U, EE, and NN) which offer definite data. In each instance the progeny of the exconjugant survived while the parent culture died (figs. 12, 14, 15, and 25). And this evidence, direct as it is, has been amply corroborated in a very practical way by all the other work on Spathidium which for more than two years has been carried on in this laboratory. Cultures about to die have been continued with exconjugants.

It is clear, therefore, from the evidence presented above and from other data secured in this laboratory since Spathidium has been under experimentation, that conjugation, in the great majority of cases, has a direct survival value.

3. Generations of cultures. The relation of conjugation to the continuance of the life of the pedigree race from the parental to the F_5 generation under cultural conditions can be best illustrated by a specific example. From the parental culture I, at the thirtieth generation, Aa (F_1 gen.) was derived; from Aa at the 104th generation, Db (F_2 gen.); from Db at the 289th generation, EEb (F_3 gen.); from EEb at the 150th generation, TTa (F_4 gen.); and from TTa at the 80th generation, YYa (F_5 gen.). YYa died at the 300th generation.

Thus from the parental culture I to the death of the F_5 generation the series of pedigree cultures extended from November 5, 1920, to November 5, 1921—exactly a year—and the series could, without doubt, have been continued indefinitely by the same procedure. During the year the actual number of 'linear' cell divisions from the parental culture through the F_5 generation of this series was 953. Since, under the conditions of the experiment, the parental culture died after 234 divisions on February 11, 1921, it is obvious that the life of the series, by five 'conjugations' was prolonged approximately nine months, and exactly 719 divisions. These

same data may be similarly analyzed for the F_1, F_2, etc., generations of other groups, but it is unnecessary to do so here, since all the details are available in tables 1 and 2 for the reader to consider as he will. Table 3, however, summarizes the facts from a representative series of cultures, including all carried through the F_4 generation, and, therefore, the series just cited as an example.

TABLE 3

PARENT	F1	F2	F3	F4	F5	TOTAL DIVISIONS WHEN STOPPED
I–30	Aa–104	Da–205	Lc– 27	Pa– 67		433
I–30	Aa–104	Da–205	Lc– 27	Pb– 68		434
I–30	Aa–104	Da–205	Ld– 69	Ua– 49		457
I–30	Aa–104	Da–205	Ld– 69	Ub– 50		458
I–30	Aa–104	Da–205	Ld– 69	Uc– 49		457
I–30	Aa–104	Da–205	Ld– 69	Ud– 53		461
I–30	Aa–104	Db–289	EEb–150	TTa– 80	YYa–300	953
I–30	Aa–104	Db–289	EEb–150	TTa– 80	YYc– 59	712
I–30	Aa–104	Db–289	EEb–150	TTa– 80	YYe– 60	713
I–30	Aa–178	Fa–224	Ta–16	Yb– 40		488
I–30	Aa–178	Fa–224	Ta–16	Yc– 45		493
I–30	Aa–178	Fa–224	Ta–16	Yd– 47		495
I–30	Aa–178	Fb–131	Ma–106	HHa– 77		522
I–30	Aa–178	Fb–131	Ma–106	HHb– 81		526
I–30	Aa–178	Fb–131	Ma–106	HHc– 82		527
I–30	Aa–178	Fb–131	Ma–106	HHd– 82		527
I–30	Aa–267	Ga– 43	Ka–217	ZZb– 32		589
I–30	Aa–295	Ja– 25	Nb–122	GGa–202		674
I–30	Aa–295	Ja– 25	Nb–122	GGb–190		662
I–30	Aa–295	Ja– 25	Nb–122	GGc–179		651
I–30	Aa–295	Ja– 25	Nb–122	GGd–190		662
I–30	Aa–295	Ja– 25	Nb–122	KKb– 3	KK_1a– 79	554
I–30	Aa–295	Ja– 25	Nb–122	KKb– 3	KK_1b– 78	553

In brief, nothing can obscure the fact that under identical culture conditions 'new' cultures, derived from conjugating pairs in the 'old' culture, consistently survived the parent cultures. This, it appears, is a further definite demonstration of the survival value of conjugation.

4. Cumulative effects. The gradual 'building up' of the division rate of a series through successive conjugations is

most readily shown by reference to figures 8, 9, and 14, which give the graphs of the division rate of the series consisting of cultures I (parental) through Aa (F_1 gen.), Db (F_2 gen.), EEb (F_3 gen.), TTa (F_4 gen.), and YYa (F_5 gen.). It is evident that:

1. Culture I (parental) started with an initial rate of slightly above four divisions per day which was never again attained throughout its life (fig. 8).

2. The Aa culture (F_1 generation) started with an exconjugant from culture I at its 30th generation, attained but never exceeded the division rate of its parent (fig. 8).

3. The Db culture (F_2 generation) started with an exconjugant from Aa at its 104th generation, never attained the maximum of its parental and grandparental cultures (fig. 9).

4. The EEb culture (F_3 generation) started with an exconjugant from Db at its 289th generation when it was dividing less than one and one-half divisions per day and rapidly approaching extinction, exhibited a restoration of division energy which reached three and one-half divisions per day. However, this is below the initial maximum of the P, F_1 and F_2 generations (fig. 14).

5. The TTa culture (F_4 generation) started with an exconjugant from EEb at its 150th generation when dividing approximately twice per day, immediately attained and maintained (until discontinued) a rate of nearly four divisions per day. This is almost as high as the maximum of the P and F_1 generations, identical with the initial maximum of the F_2 generation, and much higher than that ever reached by the F_3 generation (fig. 14).

6. The YYa culture (F_5 generation) started with an exconjugant from TTa at its 80th generation when dividing at an average rate of nearly four times per day, exhibited an initial rate of nearly four and one-half divisions daily, the grand maximum for the entire series (fig. 14).

The conclusions from this pedigree series from the P through the F_5 generation are obvious. Granting that the series selected as our example is the most striking case the

data afford, the fact remains that the other cultures, directly involved in the specific experiment to 'build up' the division rate to a maximum equal or better than that ever exhibited before by this pedigree race of Spathidium, consistently show a similar cumulative effect of conjugation, indeed, the series culminating in group XX reached a still higher maximum.

Accordingly, all the data involved corroborate the culture I through YYa series and show that it is possible by successive 'conjugations' progressively to increase, within limits, the maximum division rate.

D. Effect of close inbreeding

Throughout all these experiments the animals employed were descendants from the one 'wild' Spathidium isolated to start the parent culture I. Therefore each generation (F_1 to F_5) in the series was the product of close inbreeding. As a matter of fact, the two animals which conjugated in no instance were more than eight divisions removed from a common ancestor.

Since, as has been shown, conjugation effected an equal, if not greater, acceleration of the reproductive rate during the later experiments, it follows that continued close inbreeding, at least to the F_6 generation, does not significantly alter the typical physiological effect following conjugation.

In order to compare exconjugant cultures arising by such close inbreeding with similar cultures arising from the crossing of diverse strains, an attempt was made to secure a 'wild' race of Spathidium, but all efforts failed. We then attempted to cross animals, from our cultures, which were separated by a large number of generations. The difficulties involved resulted in only one experiment being satisfactorily carried through to completion. However, although it is not possible to draw any significant conclusion from a single experiment, it will be described briefly, since it shows that this method of attack on the question offers possibilities for valuable results with an organism such as Spathidium which conjugates readily.

A single animal from two selected cultures, in which conjugation was actively proceeding in the stock, were placed together in as small a drop of culture fluid as possible in order to increase the chances of the two meeting and uniting. No Colpidia were supplied for food. Such preparations were then examined every few hours to determine if evaporation had reduced the fluid below the minimum necessary for life and whether either had divided or conjugation had taken place. If necessary, a tiny amount of culture fluid was added. If division had occurred, the preparation was useless, and accordingly discarded.

Obviously, the chances of all the necessary conditions being fulfilled are slight—not the least among them being the actual cross-conjugation. As a matter of fact, more than 400 attempts were made and only one was successful. This was a cross between an animal from Ga at the 275th generation and an animal from CCc at the 105th generation on May 18, 1921. As table 1 and figure 7 show, Ga was the F_2 generation from the stem culture I through Aa. At the date of crossing Ga was 549 divisions removed from culture I. Similarly, CCc was the F_3 generation from culture I through Ea and Xa, and at the date of crossing was 314 divisions removed from culture I. Culture Ea arose from culture I, 119 divisions later than culture Aa. Therefore, two animals mated were actually 982 divisions removed from a single common ancestral animal.

Synchronously with this 'cross-culture,' four controls were started: two pairs of conjugants from each of the two parent cultures (Ga and CCc), so that the entire experiment comprised (in addition to Ga and CCc) Ga_2 (from Ga), CCc_2 (from CCc) and the 'cross-culture' GC.

The result from all these cultures, involving forty-four animals whose division rate was recorded daily for twenty-five days, showed no significant difference in rate of reproduction between the progeny of conjugants which were not more than eight divisions (about forty-eight hours) removed from a common ancestor, and the progeny of the cross-conjugants, the members of which were removed 982 divisions (185 days) during which five 'conjugations' were interpolated.

Although no special weight should be given to this result, so far as it goes, it is in accord with the data in regard to inbreeding from our other cultures, viz., close inbreeding for successive generations at least to the F_6 generation, apparently does not significantly alter the physiological effects which follow conjugation at the F_1 generation.

SUMMARY

The study of a large series of pedigree cultures of Spathidium spathula, all derived from a single 'wild' specimen, involving a comparison of the division rate and longevity of cultures, which had recently conjugated, with their respective parental cultures, which had not so recently conjugated, justifies, we believe, the following conclusions:

1. Spathidium can live indefinitely under entirely favorable environmental conditions without endomixis or conjugation.
2. Conjugation in the majority of cases directly induces an immediate acceleration of the rate of reproduction.
3. Conjugation is most effective in accelerating the rate of reproduction when the latter is low.
4. The accelerated division rate of the exconjugant culture in the majority of cases persists at least as long as the life of the parent (non-conjugant) culture.
5. Exconjugant cultures in the majority of cases, under the conditions of the experiment, outlive their parent (non-conjugant) cultures.
6. Conjugation typically has a high survival value in the life of the organism.
7. By permitting conjugation to occur successively, at opportune periods, the rate of reproduction may be gradually 'built up' to a higher 'standard.'
8. Inbreeding to the F_6 generation exhibited no deleterious effects.

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219 **(2451)**

Observations on the cellular activity in a culture of amphibian liver tissue.

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On January 2, 1924, two, very large, ameboid cells appeared in a culture of liver tissue, obtained from an adult *Rana pipiens*. This culture had been made on November 25, 1923, some five and one-half weeks earlier, and no cells had previously migrated into the clot. The culture was one of a series of twenty-five, and in none of the others was any cellular activity ever observed. The unusual size and activity of these cells, as well as their migration into the clot after the long inactive period of the tissue immediately commanded attention, and careful observations were made during the succeeding five days in which they retained their activity.

Each of the cell bodies was very irregular and constantly changing in shape, due to a flowing movement of the cytoplasm strikingly similar to that in an ameba. As a result of the cytoplasmic flow, cell processes of various lengths were continually formed at various regions of the periphery. When first observed each of the cells measured about 0.33 mm. in length. Later a maximum length of over 0.5 mm. was attained by one of the cells. The cytoplasm contained many granules of various sizes which moved into or out of the processes with the cytoplasmic current. After five days of continual activity in the clot, both cells rounded off, and each formed a somewhat spherical cell

body which measured about 0.15 mm. in diameter. No further movement occurred in either of these cells although the culture was opened and one of them was isolated and placed in fresh medium in a subculture.

The identity of these cells is not clear, and so far as I am aware similar metazoan cells have not been described either in tissue cultures or elsewhere. During the past few years I have observed over a thousand cultures of various adult amphibian tissues, in none of which have cells of this type been present. Also a great many permanent histological preparations of various amphibian tissues have been studied with the same negative result. The possibility that they might be embryonic in type suggested itself and accordingly a large number of cultures of various tissues from the tadpole have been made and studied. In several of these, cells comparable in size and activity have been found. The experiments are still in progress.

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218 **(2450)**

Autoplastic and homoplastic parathyroid transplantation.

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Six cats were thyroparathyroidectomized and autoplastically grafted with the two external parathyroids. In four animals the glands were implanted into a pocket cut in the sternohyoid muscle; the remaining two cats were transplanted in the *rectus abdominis* muscle. The parathyroids were dissected free from all investing membranes, the dissection being done in a half and half mixture of warm, sterile Ringer's solution and blood serum of the animal from which the glands were taken. In some instances the parathyroids remained in the solution for fifteen minutes before implantation. Table I summarizes the principal results of the experiment.

In a series of homoplastic transplantations, twenty-eight cats were used, the donor of the glands being of the same sex as the recipient, and as nearly the same age as possible. The grafts were of two types with respect to the time when made: (1) immediately following the onset of tetany symptoms; (2) simultaneous or criss-cross operations in which the parathyroids of two cats were exchanged. Tables II, III, IV summarize the chief results of the various types of operation.

TABLE I.

Cat	Location of graft	Symptoms following thyroparathyroidectomy	Recovery	Reextirpation of graft	Result	Remarks
1	Rectus abdominis muscle	Tetany	Complete	36 days after implantation	Fatal tetany	Graft in good condition. Central area replaced by connective tissue.
2	Sternohyoid muscle	Tetany	Complete	33 days after implantation	Fatal tetany	Graft in good condition. Both glands healed in. Central area replaced by connective tissue.
3	Sternohyoid muscle	None	Complete	31 days after implantation	No symptoms	Graft in good condition. Central area replaced by connective tissue. Accessory glands found at autopsy.
4	Sternohyoid muscle	Tetany	Complete	37 days after implantation	No symptoms	Graft in good condition. Central area replaced by connective tissue. Accessory parathyroids probably present.
5	Rectus abdominis muscle	Tetany	Complete	34 days after implantation	Chronic tetany	Graft tissue well preserved. Central area as in other cases. Small accessory parathyroids found in thymus.
6	Sternohyoid muscle	None	Complete	30 days after implantation Glands not found	No symptoms	Graft not located.

TABLE II.
Grafts of the parathyroid into the ear following the onset of tetany.

Cat	Sex	Weight	Symptoms after thyroparathyroidectomy	Grafted	Symptoms	Result	Duration of experiment after grafting	Weight	Remarks
		gm.					days	gm.	
34	♀	550	Tetany and depression	5th day after operation	Tetany and depression	Death	4	450	Graft degenerate. Little vascular connection.
35	♀	820	Tetany and depression	3rd day after operation	Tetany and depression	Death	4	560	Degeneration of graft. Poor vascularization.
37	♀	2250	Tetany	4th day after operation	Tetany	Death	6	1630	Graft degenerate.
38	♀	600	Tetany and depression	4th day after grafting	Tetany and depression	Death	5	502	Graft degenerate.
41	♂	2600	Tetany	7th day after operation	Tetany	Death	6	1000	Graft degenerate.
42	♂	1801	Tetany	2 days after operation	Tetany	Death	4	1740	Graft degenerate.

TABLE III.
Grafts into the Rectus abdominis muscle after onset of tetany.

Cat	Sex	Weight	Symptoms after thyroparathyroidectomy.	Grafted	Symptoms	Result	Duration of experiment after grafting	Weight	Remarks
		gm.					days	gm.	
50	♂	320	Tetany	24 hours after operation	Tetany and depression	Death	3	290	Degeneration of the graft.
52	♀	420	Tetany	24 hours after operation	Tetany	Death	2	408	Graft not sectioned.
64	♀	2935	Tetany	2 days after operation	Tetany	Death	10	2250	Degeneration of graft.
65	♂	2010	Tetany	24 hours after operation	Tetany	Death	4	1800	Degeneration of graft.
66	♂	2001	Tetany	2 days after operation	Slight tetany	Recovery	32*	1979	Part of graft persisted in fair condition.
68	♂	3660	Tetany	2 days after operation	Tetany and depression	Recovery	36*	3400	Degeneration of graft. Accessory parathyroids probably present.

*Animal succumbed to adrenal insufficiency due to removal of both adrenals. This accounts for the weight loss.

TABLE IV.

Transplantation of parathyroids into the Rectus abdominis muscle before the onset of tetany.

Cat	Sex	Weight	Symptoms	Result	Duration of experiment	Weight	Remarks
		gm.			days	gm.	
53	♂	650	Depression	Death	3	600	Graft poorly vascularized. Not sectioned.
54	♀	1510	Tetany	Death	7	1205	Degeneration of the grafted glands.
55	♀	2420	Slight tetany	Recovery*	Graft removed after 21 days. No symptoms	2020	Parts of grafted glands persisted in fair condition. Accessory parathyroids probably present.
56	♂	5050	No symptoms	Recovery	22	4786	Degeneration of graft.
57	♂	3240	Tetany	Recovery	20	3915	Degeneration of graft. Small accessory parathyroid found in thoracic thymus.
58	♂	3011	No symptoms	Recovery	Graft removed after 10 days. No symptoms	3024	Degeneration of graft. Peripheral border in fair condition.
59	♂	3565	Tetany	Death	7	3430	Degeneration of graft.
60	♂	1210	No symptoms	Recovery	12	1314	Degeneration of graft. Accessory parathyroid found in thymus.
61	♂	2540	Tetany	Recovery	30	2601	Degeneration of graft. Accessory parathyroid tissue not found.
69	♀	3160	No symptoms	Recovery	30	2540	Parts of both grafted glands persisted in fair condition.
70	♂	3120	Tetany	Death	3	3020	Graft not sectioned.
71	♂	2750	Tetany	Death	4	2610	Degeneration of entire graft. Gland area well vascularized.
72	♀	3420	Tetany	Death	4	3360	Grafts made subcutaneously on account of pregnancy. Could not locate the grafts later.
73	♀	1824	Tetany	Death	4	1773	Great degeneration of the grafted tissue. Glands well vascularized.
74	♀	2700	Tetany	Death	7	2100	Degeneration of central area of graft. Peripheral area persisted in fair condition.
75	♀	2250	No symptoms	Recovery	45	2478	Parts of both glands in fair condition.

* It is probable that most of the cats which recovered had accessory parathyroids.

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ON THE LONGEVITY OF SPATHIDIUM SPATHULA WITHOUT ENDOMIXIS OR CONJUGATION

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Studies on representatives of the various groups of Protozoa have served to emphasize the nicety of the adaptation of species and races to particular environmental conditions. Even such relatively ubiquitous forms as *Paramecium aurelia* and *Paramecium caudatum*, which are able to survive temporarily under very diverse conditions, gradually succumb to prolonged subjection to media which at first seem entirely suitable. Ample evidence of this is afforded by the history, during more than four score years, of the study of Paramecia cultures, since increased refinements of culture methods have extended the apparent potentialities of life from a few days and generations to more than fifteen years and nearly as many thousand generations in *Paramecium aurelia*—in fact, we may say, indefinitely. And this has relegated the so-called "cycle" of this organism to the realm of fancy.[1]

It would seem that a quite similar history has been briefly recapitulated in this laboratory in the studies on *Spathidium spathula* during the past four years. The majority of the cultures carried by Woodruff and Spencer showed what heretofore would have been interpreted as "cycles" which culminated in death unless conjugation was permitted, though certain cultures continued to live for over 500 generations without conjugation or endomixis. The drift toward longer life was so evident as more and more experience was gained in culturing the animals, that Woodruff and Spencer and Moore interpreted the data as clearly indicating the ability of Spathidium to live indefinitely without conjugation or endomixis under entirely suitable cultural conditions.[2] This conclusion is so far-reaching in its import for the interpretation of the life histories of the Protozoa in general, and the Infusoria in particular,[3] that we have investigated it further in Spathidium. The present paper gives a brief statement of the results.

The animals employed in this work were derived from an excysted Spathidium that was isolated from a laboratory aquarium into which had been put, from time to time, the Spathidia discarded from the pedigree cultures under observation. Presumably, indeed almost certainly, the excysted animal was a descendant of those used in previous experiments.

The definitive pedigree lines were derived from one animal of a pair of conjugants among the descendants of the animal above mentioned. The four lines comprising the culture were carried on by daily isolation in standard beef extract, with Colpidia as food. For details of the method.

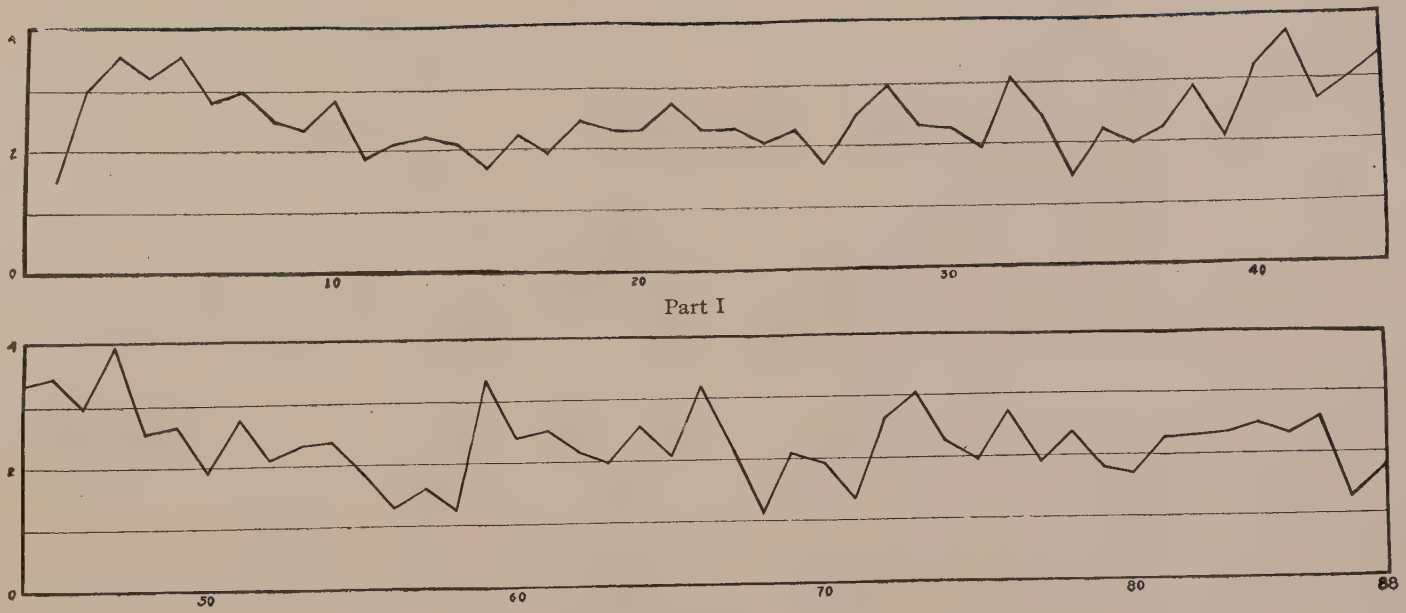

FIGURE I

Graph of the rate of reproduction of a pedigree race of *Spathidium spathula* during 1088 generations. The ordinates represent the average daily rate of division of the four lines of the culture, again averaged for five-day periods. The figures below (10, 20, etc.) indicate the numbers of the five-day periods.

reference may be made to our previous papers. During the early part of its history the culture was employed for a series of experiments,[4] and after their completion it was continued for the study of longevity without endomixis or conjugation.

The culture was carried from October 12, 1922, to December 30, 1923, when it was lost. Its life, therefore, comprised 444 days during which time 1080 generations were attained without endomixis or conjugation.

Inspection of the accompanying graph, giving the average daily division rate of the four lines of the culture throughout the 88 five-day periods of its life, shows the usual fluctuations, which are due, in part, to unavoidable environmental changes in temperature and food supply. From five-day period 36 to the end, no attempt was made to keep the culture conditions constant. Though beef extract was continued as the basic culture medium the Colpidia were taken from various sources and Colpoda were also sometimes present in the food supply. In period 53 the culture was transferred from New Haven to the Woods Hole Marine Biological Laboratory, and in period 65 it was brought back to New Haven. Following each of these transfers considerable difficulty was experienced in getting the culture established. All of these changes are registered in the rate of division. The graph gives no indication of depression periods which can be interpreted as "cycles," or of a continuous, gradual decline in reproductive activity as the culture proceeds. The slightly higher rate during the early periods is undoubtedly accounted for by the extreme precautions which were then taken with the food supply since, as previously stated, the culture was being used in other experiments.

But the culture was terminated by death in period 88, which naturally raises the question whether or no this was the result of inherent exhaustion due to long multiplication without endomixis or conjugation. All the evidence points unequivocally to a negative answer. The culture up to period 88 was continuing the even tenor of its way, when the animals suddenly appeared abnormal and all four lines died within two days without further division. Apparently it is just such an incident as occurs at intervals in all extensive pedigree work with Infusoria, and had occurred before in the present work. Previously, however, this culture had not been exterminated because the main lines were reëstablished from subsidiary lines started daily and specifically carried as "stock" for emergencies of this kind. In the present instance, owing to the pressure of other experimental work, this precaution unfortunately had been omitted, and fatal results followed.

In brief, our experience in this laboratory with pedigree cultures of Spathidium has shown progressive lengthening of life, without endomixis or conjugation, as the subtle conditions necessary for the welfare of this highly specialized species have been more nearly attained. The present

culture has demonstrated, at least, that more than one thousand generations may be attained, and makes practically positive the conclusion that *Spathidium spathula* can reproduce indefinitely without recourse to endomixis or conjugation when entirely suitable environmental conditions are supplied.

[1] Dujardin: *Histoire naturelle des Zoophytes: Infusoires*, Paris, 1841; Maupas: "Recherches expérimentales sur la multiplication des Infusoires ciliés," *Arch. zool.ex péru. gén.*, 2me sér., **6**, 1888; Calkins: "Death of the A-series of Paramecium Caudatum; Conclusions," *J. Exper. Zool.*, **1**, 1904; Woodruff: Two Thousand Generations of Paramecium, *Arch. Protistenk.*, **21**, 1911; Woodruff: "The Present Status of the Long-continued Pedigree Culture of Paramecium Aurelia at Yale University," *Proc. Nat. Acad. Sci.*, **7**, 1921.

[2] Woodruff and Spencer: "Studies on Spathidium Spathula, II, The Significance of Conjugation," *J. Exper. Zool.*, **39**, 1924. Moore: "Endomixis and Encystment in Spathidium Spathula." *Ibid.*, **39**, 1924.

[3] Calkins: "Uroleptus Mobilis. III. A Study of Vitality," *J. Exper. Zool.*, **31**, 1920. Calkins: "What did Maupas Mean?" *Amer. Nat.*, **57**, 1923.

[4] Moore: "A Further Contribution to the Study of the Effects of Conjugation and Encystment in Spathidium Spathula," *J. Exper. Zool.* (in press).

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NEUROBLAST VERSUS SHEATH CELL IN THE DEVELOPMENT OF PERIPHERAL NERVES [1]

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FIVE PLATES AND TWENTY-SIX FIGURES IN TEXT

CONTENTS

Introduction 124
Observations on normal embryos 129
1. Early development of spinal nerves 129
2. Sensory nerves in the fin folds 140
3. Observations on the behavior of the Schwann sheath cells in the living organism 142
Experimental 144
1. Removal of the sheath cells, the ganglion cells remaining intact 147
a. The effect of the removal of the ganglion crest in the trunk region 147
b. The effect of removing the ganglion crest and the placodes of the head 165
2. Experiments in eliminating ganglion cells while retaining the sheath cells 170
a. Effect of removal of the ventral half of the medullary cord.... 170
b. Effect of removal of the medullary cord after the spinal ganglia have begun their development 175
c. Effect of removal of the vagus ganglion on the r. lateralis vagi.. 178
Discussion 180
Summary 185
Literature cited 188

[1] It was hoped that this paper might appear in the volume published in honor of Professor Ramón y Cajal. Unavoidable delay in its preparation made this impossible, but the author wishes, nevertheless, to offer it here as a tribute to that great investigator, whose work has marked an epoch in the science of neurology and has ever been a source of stimulus and encouragement to him.

INTRODUCTION

One who follows historically the subject of the histogenesis of the nervous system cannot but be impressed by the deviousness of the path of progress. Nearly sixty years ago the lines were already drawn for the three-cornered controversy which has waged till the present day, now in favor of one, now of another party, but at no time set at rest by unanimity of opinion. In the "Mikroskopische Untersuchungen" Schwann ('39) first set forth the cell-chain theory, which was followed in turn by the outgrowth theory of Bidder and Kupffer ('57) and finally by the protoplasmic-bridge theory of Hensen ('64). These three concepts are of course not altogether mutually exclusive, and different shades of opinion have been held with regard to them, but nothing of a radically different nature has yet been proposed.

With the foundation of the neurone theory by His ('86) and Forel ('87), the study of the nervous system entered upon its modern phase. The investigations of His ('86, '87, '88, '90), followed by the work of Ramón y Cajal ('90, '92, '94), who first applied the Golgi method to the study of the embryonic nervous system, and that of v. Lenhossék ('92, '95), gave a firm footing to the concept of the neurone by showing clearly, as it then seemed, that each nerve fiber arises as the outgrowth of a single ganglion cell. Nevertheless, the theory of the pluricellular or syncytial origin of the peripheral nerve fiber found a considerable group of adherents,[2] but it was due particularly to the work of Apáthy ('89, '97) on the nervous system of leeches that opposition to His's views became so general a little later. One has only to refer to the studies upon this subject, published in the first decade of the present century, to realize the weight of opinion at that time against the outgrowth theory and the neurone concept sensu stricto.[3]

[2] Cf. Hoffman ('83, '89), Beard ('89, '96), Sedgwick ('95), and Platt ('96).

[3] Cf. Raffaele ('00), Dohrn ('01), Nissl ('03), Hensen ('03), Bethe ('03, '06), Kerr ('04), LaPegna ('04), Fragnito ('04, '05), Joris ('04), Brachet ('05), Braus ('04, '05), O. Schultze ('04, '05, '06, '08), Kohn ('05), Held ('06, '09), and Paton ('07).

All through this period, however, evidence was accumulating on the opposite side, and recent opinion has again become more and more favorable to the outgrowth theory.[4]

It has been the same with regard to the question of the regeneration of peripheral nerves. Particularly under the influence of Bethe ('03, '07, '08) the polygenetic theory of the regeneration of the axone found many advocates. Formidable evidence against this view has nevertheless accumulated steadily,[5] so that now relatively few[6] still hold that the cells of the sheath of Schwann, which are activated by trauma to the nerve, actually form an integral part of axis cylinders of the regenerating fibers.

At the time when these controversies were at their height, the present writer ('04), realizing the difficulties of settling the important questions at issue by purely histological methods, even by the very delicate ones then in use, sought to apply experimental methods to the study of the histogenesis of peripheral nerves in the embryo.

It was found in the course of this work, that after removal of the ganglion crest of young amphibian embryos peripheral motor nerves, in the form of naked axis cylinders, developed in place of nerves which normally had numerous spindle-shaped sheath cells about them. This and other considerations led to the inevitable conclusion that these cells, which by many had been regarded as giving origin to the peripheral axones, were, as far as the development of the latter structures are concerned, merely of an accessory nature. The experiments leading to this conclusion have recently been repeated by Müller and Ingvar ('21) with like results.

[4] Cf. Harrison ('01, '04, '06, '10), Neal ('03, '14, '21), Forel ('05), v. Kölliker ('05), v. Lenhossék ('06), Mott ('09), Hoven ('10), E. Müller ('13), the later work of Dohrn ('07), and Braus ('11), in which views previously held were in part modified, and the studies of Ramón y Cajal ('06, '07, '08, '13, '20) and Tello ('17, '23) with the use of the silver method.

[5] Cf. Langley and Anderson ('02, '04), Ramón y Cajal ('05, '07, '13), Münzer ('06), Lugaro ('06), Perroncito ('07), Tello ('07), Dustin ('10, '17), Bersou ('13), Boeke ('15, '16), Nageotte ('22), and others.

[6] Cf. Barfurth ('05), Walter ('08), and Henriksen ('21).

Other experiments,[7] with hanging-drop cultures of embryonic nervous tissue in clotted lymph, rendered possible the direct observation of the growing nerve fiber, showing the latter to be not a chain of cells, but a strand of hyaline protoplasm with an active amoeboid end, through the motion of which the fiber is spun.[8] In other words, it was actually seen that the nerve fiber grows as the process of a single neuroblast. Like the sheath cells, the protoplasmic bridges (plasmodesmata) are therefore not essential to the formation of peripheral axones.

While these results have been widely accepted, a considerable number of writers still adhere either to the Schwann-Balfour cell-chain theory or to Hensen's protoplasmic-bridge theory. The evidence against the latter has been fully discussed in the papers just cited, but inasmuch as the experimental evidence against the cell-chain theory has never been given in detail, it is planned to do so in the present communication, even though the conclusions but confirm those already reached.

Previous work on the histogenesis of the nerve fiber will not be reviewed here in extenso.[9] However, a few of the more recent publications, dealing, for the most part, with the cranial nerves, especially the eye-muscle nerves, demand brief consideration.

Among these the papers by Neal ('14, '21) on the development of the motor spinal and eye-muscle nerves of Squalus acanthias, with their beautiful illustrations and attention to the finest cellular details, stand preeminent, affording, as they do, one of the clearest demonstrations of the outgrowth theory ever given. Carpenter's work ('06) on the oculomotor and abducent nerves of the chick, Dohrn's ('07) on the trochlearis of elasmobranchs, Gast's ('09) on the oculo-

[7] Cf. Harrison ('07, 08, '10), and Burrows ('10, '11).

[8] See also later work by Lewis ('12), Braus ('11), Ingebrigtsen ('13), Levi ('16, '17), and others.

[9] For such discussion the reader is referred to the papers of O. Schultze ('05), Held ('09), Ramón y Cajal ('13), Neal ('14), Schaeppi ('16), and Tello ('23), as representing the various points of view.

motorius of the same forms, and Belogolowy's studies ('10) on the cranial nerves of the chick, all speak strongly against the cell-chain theory. Likewise Held's observations ('09) upon embryos of all classes of vertebrates show clearly the secondary character of the union between sheath cell and nerve fiber. Finally, in amphibians the beautifully exact work of Coghill ('13, '14) and of Herrick and Coghill ('15) in correlating the reactions of embryos at various stages with the state of differentiation of their nervous system affords additional proof that the nerve fiber is formed by outgrowth from the nerve cell. Götte ('14), on the other hand, in his account of the development of the cranial nerves in fishes and amphibians, follows in the main the cell-chain theory, and maintains that peripheral nerves arise from a syncytial mass, in the constitution of which mesenchyme participates, as well as cells from ganglion crest, placodes, and medullary cord. Pedaschenko ('14), studying in Götte's laboratory the eye-muscle nerves of various vertebrates, principally reptiles, also claims that the nerve fibers are of syncytial origin and grow centripetally as well as centrifugally. The descriptions of Götte and Pedaschenko lead, however, to no unified concept of the development of the nerve fiber, as Götte himself admits (op.cit., p. 115). They are altogether at variance with the exhaustive and detailed descriptions given, e.g., in the works of Neal, Coghill, and Held, and nothing convincing with regard to processes of histogenesis is shown in their illustrations. One gains the impression from the study of their figures that attention to finer details, which would make it possible to discriminate between the fortuitous and the essential in the relation of growing nerves to the central and the surrounding cells, might well have led to other conclusions.

Recently Dart and Shellshear ('22) have made an attempt to overthrow the outgrowth theory, if not, indeed, all of our accepted views on the development of the nervous system. These authors, who hold in general to the Hensen theory, claim to have found that the motor nerve cells take origin in the myotomes in elasmobranch and amphibian embryos and

migrate later into the medullary cord. What they have actually observed, if one may judge by their figures, is merely the development of the sclerotomes—structures which have nothing to do with the nervous system. Their paper is essentially critical, and they are very exacting in their demands upon others for the most rigorous kind of proof, but they offer nothing of that nature in their own communication. Moreover, they attach undue weight to certain irrelevant or inconclusive evidence, at the same time ignoring the detailed findings in such studies as those of Neal, which are much more convincing.

To sum up the impression gained from the study of the relevant literature accumulated since 1900, we find that the neurone concept, in so far as it applies to the development and regeneration of the nervous system, remains unshaken under the attacks it has received, and that it now stands more firmly established than ever.

In proceeding now to the consideration of the particular part played by the present investigation in leading to this conclusion, it should be borne in mind that the problem before us is not how the nerve fibers reach their destination and assume their final arrangement with respect to the other organs and tissues of the body, but simply how the axone is constituted in development—whether it takes origin from a cell chain or is the process of a single ganglion cell. The experiments deal with the behavior of these two classes of cells under varied conditions and lead to definite conclusions as to their respective importance in the histogenesis of the axone.

Anuran embryos were used for the experiments. The method of procedure was to eliminate by appropriate operation, first, the source of the sheath cells and, second, that of the motor ganglion cells, in one case by removing the ganglion crest, and in the other by removing the ventral half of the medullary cord. The work has of course been controlled by the study of normal embryological material supplemented by the examination of the nerves of the tail fin in Triton and

Amblystoma larvae. The observations agree very closely with those of Held ('09), who also opposes the cell-chain theory, but the interpretation put upon the significance of the plasmodesmata is altogether different from Held's.

Obviously, experiments of this kind may throw light on certain interesting questions other than the histogenesis of the nerve fiber, such as myelinization, the development of the sympathetic system, and the origin of the suprarenal medulla and other chromaffine tissue. In fact, Kuntz and Batson ('20), Müller and Ingvar ('21, '23), and Kuntz ('22) have already used this method in their investigations on the origin of the sympathetic ganglia; unfortunately, however, with contradictory results. These papers are discussed more fully below (p. 183, foot-note).

OBSERVATIONS ON NORMAL EMBRYOS

1. Early development of spinal nerves

Before and immediately after the closure of the medullary tube in the frog embryo, the cells in its walls are arranged in several ill-defined layers, the individual cells being somewhat elongated radially with respect to the curvature of the tube as a whole.

The stage (fig. 12)[10] at which the operations for the removal of the ganglion crest and the ventral half of the medullary cord are done is slightly older than the above. The cells of the medullary cord (fig. 27, *m.c.*) stretch out radially, and many of them now reach from the inner to the outer limiting membrane. The nuclei are likewise elongated. Certain cells (*nbl*), however, occupying the peripheral zone of the wall of the tube, remain rounded and contain round nuclei. These are the first of the neuroblasts of His, which will soon begin to form nerve fibers. Cell boundaries are indistinct at this stage except when marked by the unequal pigmentation of the cells, but it should be emphasized again here that the walls of the neural tube do not constitute a syncytium. This

[10] The figure represents an embryo of R. esculenta. The description is taken from transverse sections of an embryo of R. sylvatica (4a).

may readily be demonstrated by teasing them in the fresh condition, when the cells appear as distinct round vesicles with delicate cell membrane.[11] In embryos of this age there are as yet no nerve fibers whatever, either peripheral or central. The ganglion crest of the head is well developed, however, and has already grown far ventrally. In the anterior part of the trunk it is beginning to spread out, some of the cells reaching to the dorsal border of the muscle

Fig. 1 Rana sylvatica embryo (5a) near close of non-motile stage; some motor roots and dorsal nerves present. × 10.

Fig. 2 Rana sylvatica embryo (6a) after first muscular movement. × 10.

Fig. 3 Rana palustris embryo (7a); spinal motor roots present to the eighteenth somite. × 9.5.

Fig. 4 Rana palustris embryo (9a). × 9.5.

plates. In the middle of the trunk it rests entirely upon the medullary tube, while near the tail it can scarcely be distinguished at all.

These conditions persist for a short time until the tail bud becomes more prominent (fig. 1). Then certain internal changes take place, which mark the beginning of histological differentiation in the nerve elements. It is difficult to determine by external criteria whether actual nerve fibers are present in a given specimen of this age or not. Two individuals scarcely distinguishable superficially may differ in this regard. The first movements of the embryonic muscu-

[11] Cf. Harrison ('10), p. 793.

lature do not take place, however, until after nerve fibers are present (Hooker, '11; Coghill, '13). The most precocious nerve in its differentiation is the r. ophthalmicus profundus trigemini. Almost simultaneously with this, there develop a few of the fibers of the r. lateralis vagi, the first three or four segmental dorsal nerves from the Rohon-Beard cells, and the corresponding motor roots.

In a series of frontal sections of a Rana sylvatica embryo (5 b) 4.10 mm. long the above nerves are all present. Motor fibers are developed in connection with the second, third, fourth, and fifth myotomes. They are of particular interest in showing the primitive direction taken by the root fibers, as seen on the right side of the embryo. In the second segment there is but one fiber, and this runs transversely to the muscle plate. Two distinct fibers are present in the third segment, both taking an antero-lateral direction. In the fourth segment one very clearly defined fiber may be followed from a cell in the cord postero-laterally to the myotome (fig. 28, nf_1). A second fiber (nf_2) leaves the cord posterior to the former and crosses it at right angles.[12] Similar relations are found in the fifth segment. Frontal sections are not favorable for the study of the ganglion crest, but in all of the segments where motor roots are present, ganglion-crest cells have reached the level of these nerves, having migrated ventrally between the myotomes and the medullary cord. The motor root fibers run close to these cells, but there is no definite relation between the latter and the fibers at this stage. This embryo, when preserved, was still in the non-motile condition.

An embryo (fig. 2)[13] slightly older than the above, as shown by the increased prominence of the tail bud and the first muscular movement (contraction on the same side of the body at the point stimulated), has a greater number of dorsal nerves, probably thirteen in all, but the motor roots extend back only to the sixth somite, inclusive. Posterior to this

[12] According to Coghill ('13), the first motor fibers to develop are formed as collaterals of the fibers of central motor tract.

[13] R. sylvatica 6a, 4.4 mm. long, cut into transverse sections.

segment motor neuroblasts stand out clearly in the medullary cord, but the axone processes have not broken through the external limiting membrane.[14] The motor fibers may be traced from the cord to the muscle plates and thence along the medial border of the latter for a short distance. This specimen shows very plainly the disintegration of the neural crest. While many of its cells have crept in between the medullary cord and the myotomes, others apparently remain in the region dorsal to the cord and may enter the dorsal fin to form mesenchyme. In the anterior part of the trunk of the embryo, where the motor roots have already developed, and to some extent posterior to this region, ganglion-crest cells, as noted in the previous case, have wandered ventrally as far as the level of these nerves. From the ventro-medial border of the myotomes, about as far back as the eighth somite, segmental bands of cells, the sclerotomes, extend dorsally between myotome and notochord as far as the ventral roots. These two streams of cells meet near the ventral border of the medullary cord. At first the sclerotome cells appear rounded while the neural-crest cells are for the most part spindle-shaped; the latter are rather more heavily pigmented and contain less yolk, but where the two kinds of cells come into contact with one another, it is scarcely possible in every case to distinguish between them. The motor nerves, on breaking through the confines of the medullary cord, come into closer relation with the ganglion-crest cells than with those from the sclerotome. However, there are still no definite topographic relations between these cells and the motor axones, such as are found later when the sheath cells apply themselves closely to the latter.

A similar embryo,[15] but slightly older and cut into frontal sections, shows motor roots from the second to the ninth somites, inclusive. The fibers of a given root do not all leave

[14] Cf. Harrison ('01, pp. 370 and 408) and Neal ('21, p. 69). These observations are important since they are contradictory to the recent descriptions of Dart and Shellshear ('22).

[15] R. sylvatica 6b, 4.6 mm. long.

the cord in the same transverse plane, but are spread to a considerable extent. The fibers coming from the anterior part of the segment run obliquely posteriorly and those from the posterior part oblique anteriorly, the two converging together laterally. The mean direction of the fibers is thus transverse. The number of elements entering into a single segmental nerve at this stage is still very small, ranging from about two to four axones. At this stage distinct longitudinal bundle fibers are present in the cord. Both the sclerotomes and the ganglion crest show a well-marked segmental segregation. Sections through the middle level of the medullary cord show distinct masses of ganglion-crest cells opposite the middle part of each myotome, but intersegmentally there are none. The segmental arrangement of the spinal ganglia is thus foreshadowed from the time when the ganglion-crest cells first press in between the medullary cord and the myotomes.

Embryos slightly older (fig. 3)[16] show a more definite segregation of the ganglion-crest cells, so that we may now call them spinal ganglia. These ganglionic masses, which will later become still more compact, are found throughout the definitive trunk region and also in the segments that will enter the base of the tail. Each ganglion reaches ventrally to the space between notochord, myotome, and spinal cord, and surrounds completely the ventral root as it issues from the cord. Mitoses are frequent in the cells constituting the ganglia, which are as yet but slightly differentiated. There is some accumulation of cytoplasm and the nuclei in some cases are vesicular, but there is no trace either of dorsal root fibers or peripheral fibers originating from the ganglion cells. The only sensory nerves present in the trunk region are the dorsal nerves arising from the Rohon-Beard cells, which lie within the cord. Motor roots are present back to the eighteenth somite. The fibers in each segment converge as in the previous specimens, the general direction of each motor root, even the ones farthest back, being approximately transverse.

[16] R. palustris 7a and 7b, 4.8 mm. long.

The ganglia in a specimen 6 mm. long,[17] in which the gills are just beginning to sprout, are somewhat more compact and contain some cells more highly differentiated than before. The motor fibers may be traced to the level of the middle of the notochord, and while for the greater part of their peripheral course they pass through the ganglia, no distinct sheath cells are yet present. They are still in the primary cell-free condition (Held). At this period the spinal ganglion cells can usually be distinguished from the sclerotome cells by their lesser yolk content and by their less dense chromatin.

A little later[18] (fig. 4) the motor nerve fibers may be traced in transverse sections as far as the ventral level of the notochord and some spindle-shaped cells may be seen applied to them. The spinal ganglion cells now have large clear round nuclei.

In a sylvatica embryo[19] of approximately the same age, cut into frontal sections, the motor roots of the segments near the posterior end of the body are seen to take a more oblique course than in earlier stages. Each fiber consists of a bundle of fibrillae. On the stretch lying between the nerve cells in the cord and the ganglia no sheath cells are present.

In the next stage,[20] an embryo about 1 mm. longer than the previous one, the ganglia are further differentiated, but still there are no dorsal roots. These appear first in embryos of about 8 mm.,[21] in which the gill filaments are quite distinct (fig. 5) In embryos of this age the ventral rami of the motor nerves stand out more clearly, and sheath cells are more frequent. The dorsal roots have from the first far more sheath cells than the ventral.

Embryos of about 10 mm. in length[22] with long gill filaments and the beginning of the opercular folds (fig. 6) are

[17] R. palustris 8a, 5.7 mm. long.
[18] R. palustris 9a, 6.5 mm. long.
[19] R. sylvatica 9b, 6.3 mm. long.
[20] R. palustris 10a, 7.5 mm. long.
[21] R. palustris 11a, 8 mm. long.
[22] R. sylvatica 12b, 10 mm. long.

especially interesting in showing the shifting of the spinal ganglia and myotomes with respect to the spinal cord. In such a specimen each of the motor roots, back to the one belonging to the seventh myotome, consists of three bundles of fibers meeting at the corresponding ganglion. Each bundle is made up of smaller bundles of fibrillae arising from a single nerve cell, and constituting, therefore, an axone. These axones may be followed across the marginal veil of the cord from the motor cells, converging toward the corresponding spinal ganglion. The course of the larger bundles from the

Fig. 5 Rana palustris embryo (11a); first appearance of dorsal roots of the spinal ganglia. × 9.5.
Fig. 6 Rana sylvatica embryo (12b). × 9.

cord to the ganglion in the several segment is as follows: In the fourth somite all three bundles run anterolaterally to the ganglion, the last of the three being the most oblique. In the fifth somite on the left side the first bundle runs exactly in the transverse plane, and the two others run somewhat anteriorly; on the right side the first bundle runs slightly posteriorly, the other two anterolaterally. In the sixth somite there are three bundles, the first of which runs posterolaterally, while the other two run transversely. In the seventh somite the first two bundles run posterolaterally,

the third transversely. In the eighth and ninth somites only two bundles may be distinguished and both run posterolaterally. From this point on all of the roots run in this direction from the cord to the ganglia, and the angle with the transverse plane becomes larger and larger. In some cases two root bundles are present, in others but one.

It follows from the comparison of this embryo with the younger stages that there must be a change in the relative position of a given section of the cord with respect to the myotomes and the ganglia. The fixed plane seems to be in the region of the fifth myotome. Anterior to that the myotomes shift relatively toward the head; posteriorly, towards the tail. The latter is no doubt due to more rapid growth in the myotomes than in the cord. The spinal ganglia retain, however, their relative position with respect to the myotomes.

The material lengthening of the myotomes during the period in question is readily shown by measurement, from which it is seen also that the greater part of the increase falls upon the segments posterior to the fifth. Sections show that in embryos 4.4 to 4.8 mm. in length all of the motor roots—in the latter stage they are already present as far posteriorly as the fifteenth segment—leave the cord opposite the myotome to which they belong. However, in embryos 10 mm. long (of which the one just described is an example) the roots are considerably extended by the shifting of the myotomes. The nerve to the fifteenth segment, for instance, arises opposite the fourteenth. In later stages the myotomes shift still further distalward, so that nerve roots in the tail run posteriorly for a distance of two somites between their points of emergence and their proper ganglia.

The shifting of the myotomes is indicated in the same manner by the course of the fibers given off by the Rohon-Beard dorsal cells, which are very distinct at this stage. These fibers, it will be remembered, pass laterally through the notches between the myotomes, and are thus held fast in position by the latter. In this same specimen the nerve between the third and fourth segments runs from the cord antero-

laterally to the intermuscular septum and skin. The next one runs also anterolaterally, but diverges less from the transverse plane. The next, i.e., between segments 5 and 6, runs slightly anteriorly, but almost in a transverse plane. The next runs purely transversely and the others run posterolaterally, diverging more and more from the transverse plane the farther the origin of the nerve from the head.

The relation of the sheath cells to the motor nerve roots is somewhat variable in the different segments. The intramedullar portions of the roots, where they traverse the marginal veil, are, however, free from cells. In the more anterior segments the roots are also devoid of cells for a short distance beyond the limits of the cord, but, as the ganglia are approached, cells are more frequent. One gains the impression that the first sheath cells pass centripetally from the ganglia to cover the motor roots as well as in the opposite direction along the peripheral rami. In the segments farther back sheath cells cover the motor roots more completely.

The relation of the sheath cells to the dorsal nerves (Rohon-Beard) is quite variable in the specimen under consideration. The majority of these nerves are still naked, but those in the posterior part of the trunk have spindle-shaped cells closely applied to them. In a R. pipiens larva of the same age[23] and also in a similar specimen of R. palustris[24] the dorsal nerves are uniformly naked. The latter individual shows far fewer sheath cells on the motor roots than the sylvatica embryo just described.

In neither this stage nor the next[25] is there evidence of outward migration of medullary cells along the motor roots. Occasionally one finds isolated cells lying in the marginal veil, but they are too rare to warrant any conclusion with regard to their movement.

[23] R. virescens (pipiens) 12b, 6.5 mm. long.
[24] R. palustris 12b, 8.3 mm. long.
[25] R. palustris 13b, 9.5 mm. long.

Sagittal sections of specimens of the same age[26] show best the peripheral nerves, long stretches of which often lie in a single section. This refers particularly to the ramifications of the spinal nerves medial to the axial musculature. The relations of the sheath cells and ganglion cells to the bundles of neurofibrillae constituting the axones show very plainly (figs. 29 and 30). One feature in this relation should be emphasized. The sheath cells (*s.c.*) are actual spindle-shaped cells and not simply nuclei resting within the nerve fiber as O. Schultze ('05) maintained. In this respect my own observations are merely in accordance with those of many others, particularly those of Held ('09). The cells lie upon the nerve fibers. Their boundaries are distinct, especially when seen in profile, but they may be made out even when the cell overlies the axone. The cytoplasm is usually pale, but contains scattering pigment granules, and at this stage still may show an occasional yolk granule, though this is exceptional. Such cells may also be found associated with the spinal ganglion cells and have no doubt a common origin with the latter. It is interesting to note that this stage is younger than any specifically mentioned by O. Schultze, and yet the principal phases in the development of the peripheral axones have already been passed.

Embryos, a little older than the above (fig. 7) with external gills covered on the right but not yet on the left and with still an appreciable amount of yolk in the intestine, show the peripheral nerves to have extended to all regions of the body. In sagittal sections[27] they may be followed from the spinal ganglia to the peripheral parts of the tail fin, forming a delicate plexus just under the basement membrane. The outlying stretches of these nerves are for the most part naked axones entirely devoid of sheath cells, but toward the central portions the latter become more and more frequent. These cells are in general more extended than in earlier stages, and their boundaries are not so distinct (figs. 36 and

[26] R. palustris 12c, 9.5 mm. long.

[27] R. palustris 14c, 10 mm. long.

39). Especially when lying upon the nerve, as viewed through the microscope, it is often impossible to distinguish their outlines, and it is then that one might be misled into interpreting them as belonging to the axone itself. Their distinct character may, however, still be clearly seen when viewed in profile.

Examination of the spinal cord of embryos of this stage shows—most conveniently in frontal sections—that there are a few sporadic cells scattered about in the marginal veil in the anterior part, but practically none behind the seventh

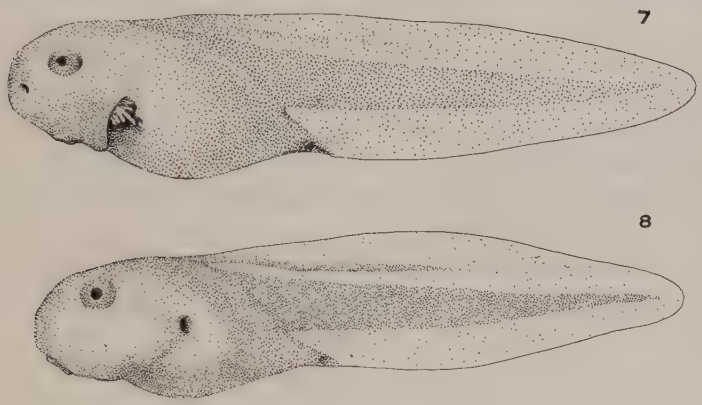

Fig. 7 Rana palustris larva (14a). × 9.
Fig. 8 Rana palustris larva (16a). × 7.

somite. Several of the cells are close to the point of exit of the motor roots.

Older specimens[28] (fig. 8) show that there is an increasing number of these cells in the anterior part of the medullary cord and that they are found farther and farther posterioly as development proceeds. Some of them are again close to the motor roots. Taken together with what is known in the teleost, elasmobranch, and cyclostome embryo, and with certain of the experiments described below (p. 160), these observations indicate that the cells in question are medullary elements, beginning their belated migration outward.

[28] R. palustris 15b, 10 mm. long, and 16a, 12 mm. long.

2. Sensory nerves in the fin folds

The fin folds of the amphibian larva have been a classical object for the study of the histogenesis of the nerve fiber, Schwann ('39), Kölliker ('46), Hensen ('64), O. Schultze ('05), and Held ('09), all having made use of it in their work. Its special significance for the present purpose was pointed out in a former paper ('04) in which it was shown that in the cutaneous nerves of the fin and adjoining portions of the skin nature had performed an experiment proving the secondary character of the relation between sheath cell and nerve fiber.

In the anuran larva there are more sheath cells on these nerves that in the urodele, they appear relatively earlier, and there are apparently no cases in which none are present. On the other hand, the distal portions of these nerves, as pointed out in connection with the last specimen described (p. 138), are without sheath cells.

In the urodele larva conditions are at first different, as shown by a study of the tail fin of Triton larvae about 10 mm. long. Eight individuals, measuring in length 8.4, 8.5 (two specimens), 9.5, 10, 10.2, 10.4, and 11.2 mm., respectively, will first be considered. The specimens, which had already been preserved, were examined in toto with the water-immersion lens. Only the largest one showed undoubted sheath cells in any nerve aside from the r. lateralis vagi. Two such cells were found in the dorsal fin and one in the ventral near the anus, and it is possible that others may be present in this individual, because pigment cells obscure the nerves in places. One other case (10 mm.) has two cells resembling sheath cells in form, but lying on a fiber-like structure which is probably not a nerve.[29]

[29] It is possible that in some cases a few sheath cells may wander peripherally at somewhat earlier stages. Schultze figures one embryo 9 mm. long showing such cells, and Held states that in Triton larvae of this length they are present. These slight discrepancies may be due to specific differences. The fact remains, nevertheless, that the nerves are at first devoid of these cells.

Some of the above individuals, as well as older ones, have been examined in sections. Besides these, others have been studied in toto after splitting the tail fin. In many cases nerves which have no sheath cells beyond the border of the myotomes do show one or two such cells between the spinal ganglia and the point where the nerves emerge from between the myotomes. In other cases, however, the nerve fibers may be followed from the ganglion to the peripheral part of the fin without showing a single sheath cell.

The main facts, as just stated, were given in the preliminary note above cited. They are repeated here because of the doubt cast upon the earlier statement by O. Schultze ('05, p. 68). It is not necessary, however, to enter further into details, since Held has confirmed the original account in the most positive manner, and has added a beautiful figure of the subepidermal nerve plexus of a 12-mm. (9 mm. according to the description in the text) axolotl larva, which shows an intricate plexus of nerve fibers without a single cell. I have seen similar plexuses in Triton, some of which were mapped before the appearance of Held's paper. In Amblystoma punctatum (maculatum) the arrangement is probably the same, but this form is not so favorable for study because of the greater number of pigment cells in the fin fold. There is one difference between Held's account of this nerve plexus and my own which should be mentioned. Held implies, though nowhere explicitly states, that this cell-free plexus has its origin in the Rohon-Beard dorsal cells. While this is no doubt partly the case, my preparations give indubitable evidence that some of the fibers arise in the spinal ganglia.

The nerves taking origin from the Rohon-Beard cells, as is now well known, give rise to fibers which form a plexus underlying the skin throughout the body of the embryo. This plexus is for the most part entirely devoid of sheath cells, and when the latter are present it is only in very late stages. The youngest Rana embryo in which I have found such cells is one of R. sylvatica (described above) 10 mm. long.

In order to ascertain the relative importance of ganglion cell and sheath cell in the formation of the sensory nerve fiber we need, therefore, but compare with one another the nerves formed by the Rohon-Beard cells in both urodeles and anurans, the nerves arising from the spinal ganglia in Triton and the corresponding nerves in Rana. In all of these cases the relation of the nerve to the ganglion cells is constant, no sensory nerves of the trunk or tail ever being found which are not connected either with spinal ganglia or Rohon-Beard cells from the beginning. On the other hand, the sheath cells, which are found early on the spinal ganglion nerves of Rana, do not appear on the corresponding nerves in Triton until after at least some of the fibers have extended through their full peripheral course, and are only occasionally present on the dorsal nerves arising from the Rohon-Beard cells.

3. Observations on the behavior of the Schwann sheath cells in the living organism

One of the points which O. Schultze emphasized in his account of the histogenesis of peripheral nerves is the intercalation of the Schwann cells in the fibers, which, according to him, constitutes a relation so intimate that the actual existence of these cells as separate entities is denied, and they are referred to merely as the 'nuclei' of the fibers (Schwann'sche Kerne). The study of these elements in the living animal shows that this view is untenable, confirming the conclusion already drawn from the study of sections.

As long known, the finer structure of the nerves of the tail fin in both anuran and urodele larvae may be readily observed in the living specimen under high powers of the microscope. For this purpose the water-immersion lens, D* of Zeiss, may be used with particular advantage. The larva is anesthetized in a chloretone solution of 0.02 to 0.03 per cent, and then, when laid on its side, the whole structure of the tail fin is open to observation. Anesthesia may be continued for hours and may be repeated from day to day, so that the same nerve fiber may be kept under continuous ob-

servation. In such specimens it is very easy to see the Schwann cells. As already noted, they are more numerous proximally and there is always a more distal stretch on which no cells are present.

The cells are very pale and are so closely applied to the fibers that in the living specimen, except in rare cases, only the nucleus is visible. Usually the nuclei are of a long oval shape, but sometimes they are irregular or they may adapt themselves to the branching of the fibers. Those found at an intersection or in the crotch of a branching nerve are usually more rounded than others (fig. 9, A, *1* and *2*).

The cells remain stationary often for long periods, as may be seen on repeated examination of the same nerve at intervals of several days or even weeks, but in general there is movement in a distal direction. Thus one case (R. esculenta larva 14.5 mm. long, fig. 9, A and B) observed eleven, sixteen, and twenty-two days, respectively, after the first observation, shows two cells (*1* and *3*) each of which moved distally during the first eleven days about 30 μ, and after that remained in place, while another cell (*2*) remained stationary the whole time. In all probability, the change of position was not due to a steady slow progression, but to a relatively rapid movement, followed by a long period of rest, as is shown by another case (fig. 10, A, B, and C) in which one cell (*2*) moved 35 μ in a half-hour and another (*1*) 14 μ in twenty-five minutes. These movements, as well as numerous others which have been observed, all took place in a proximo-distal direction. No cases of reversed movement have yet been observed.

The separateness of the sheath cells from the nerve fiber may be best seen in nerves which are undergoing degeneration after having been cut off from their central connections. The behavior of such cells may also be directly observed in the living specimens. As already described,[30] degenerative changes are very rapid in the larval anuran, the process being far advanced twenty-four hours after the nerve is cut. At this time the Schwann cells, which in the normal specimen

[30] Cf. Harrison ('08 a).

have an almost glass-clear cytoplasm, have already become markedly changed. A few highly refracting granules are present close to the nucleus (fig. 11, A and B) and many of the cells lose their smooth spindle shape and become irregular, raising themselves up from the nerve fiber. Occasionally a cell will appear entirely separated from the fiber (fig. 11, C). While the nerve is undergoing degeneration, the cells may either move or remain stationary for days, but on the whole there seems to be more locomotor activity in the cells of the degenerating nerve than in those of the normal. Even after the almost complete disappearance of the axis cylinder the cells may remain as a chain, showing the position formerly occupied by the nerve.

The above observations show clearly the fact that the sheath or Schwann cells are actual cells and not merely nuclei, and that, although intimately connected with the nerve fibers, they react independently under both normal and changed conditions.

EXPERIMENTAL

In devising a method by which the sheath cells might be eliminated, an assumption had to be made at the outset because of the uncertainty of the source of these cells in the embryo. According to one group of observers, represented by His and by v. Kölliker in his earlier papers, they are derived from the mesoderm; according to others, from the ectoderm, but within the latter layer various possible sources have been given—the ganglion crest, the ventral part of the medullary cord along the motor roots, and, in the case of the lateral-line nerves, the ectodermal thickenings which give rise to the sense organs. According to some, especially those who have advocated a mesodermal origin, the sheath cells simply appose themselves to the nerve fibers with which they come into contact, and do not carry out any very extensive migration. However, the view that they originate in the nerve centers postulates an extensive migration along the nerve trunks. The latter view seemed to afford the better working hypothesis for an experimental test.

Now it is conceivable that if the sheath cells actually do wander out from the nerve centers, some chemical means might be found to inhibit this movement. As, however, there

Fig. 9 Nerves from the dorsal fin fold of a living Rana esculenta larva 14.5 mm. long (no. 3). A, as observed July 5; B, July 16. Sheath cell *1* has moved distally ca. 30 μ, *2* has remained stationary, *3* has also moved ca. 30 μ. Obj. Zeiss D*; × 242.

Fig. 10 Nerves from the dorsal fin fold of a living Rana esculenta larva (no. 4). A, July 17, 11.35 A.M.; B, 12.05 P.M.; C, 12.30 P.M. Sheath cell *1* remained stationary during the first interval and moved about 14 μ during the second (twenty-five minutes). Cell *2* moved about 35 μ during the first half-hour and then remained stationary. Obj. Zeiss D*. × 242.

Fig. 11 Schwann cells from nerve fiber, degenerating after having been cut. From free-hand sketches. A and B, one day after cutting nerve; C, two days after. Experiment CN. 35.

was but little to guide one in seeking such a substance, it seemed more feasible to attempt the elimination of the sheath cells by a surgical operation. Accordingly, the most likely source of these elements was excised. This, in the case of the spinal nerves, seemed to be the ganglion crest; in the case of the cranial nerves, the proper placodes had to be removed as well. The removal of these structures, which at the same time are the presumptive source of the spinal and cranial ganglia and the sensory nerves, would necessarily preclude obtaining the desired evidence from this quarter. Not so, however, with the motor nerves, which run out from the ventral half of the medullary cord. If, then, after excision of the ganglion crest these nerves should develop as naked axones, the conclusion would be justified that the axones were derived from neuroblasts situated within the ventral portion of the cord and the sheath cells from the same source as those of the sensory nerves—the ganglion crest. If, on the other hand, motor nerves as well as sensory should be found lacking, then the experiment would speak for the pluricellular origin of the axone from the so-called sheath cells.

There was one serious ground for doubt about the success of this experiment and that was the possible origin of the sheath cells of the motor nerves from the ventral part of the medullary cord. This has indeed been described by a considerable number of competent observers, more especially in the cyclostomes and elasmobranchs. It has been shown by Balfour ('75), van Wijhe ('88), Dohrn ('88, '91 a), v. Kupffer ('94, p. 68), and more recently by Neal ('03, '14, '21) that cells in considerable numbers wander out of the medullary cord along the motor roots. On the other hand, my own observations ('01) on the salmon embryo indicated that in this form fewer cells take this course and that the process does not begin until relatively late. In the anuran embryo no evidence of such migration in the spinal region could be found up till the time when the yolk is nearly absorbed, i.e., not for nearly a week following the first appearance of the motor roots; and during this period the peripheral nerves

extend to all parts of the body. In view of these last observations, it, therefore, seemed probable that the source of the sheath cells of the motor nerves in the amphibians was the same as that of the sensory nerves, viz., the ganglion crest, and different from the seat of origin of the motor nerve cells, which are situated in the ventral part of the medullary cord. The projected experiment should, therefore, throw light on the question at issue. The results have shown that the expectation was not without ground.

The whole question of the migration of cells from the medullary cord along the motor roots has been considered very fully by Held ('09) subsequently to the first publication of these experiments. His results show that their source is not the same in all vertebrates, and that, in harmony with the findings of previous investigators, there is an abundant outwandering of such cells from the ventral part of the cord in cyclostomes and elasmobranchs, more sparing in the teleosts, and none at all in the stages in question in the amphibians. In all of these forms, however, the motor nerves pass through a primary stage of greater or less duration in which sheath cells are not present. Nevertheless, as Held points out (op.cit., p. 142), the present experiment would probably not have succeeded in any of these forms but the amphibians.

1. Removal of the sheath cells, the ganglion cells remaining intact

a. The effect of the removal of the ganglion crest in the trunk region. The first experiments of this series were made in the Anatomical Institute at Bonn with embryos of Rana esculenta. Later experiments were made at Baltimore and at New Haven with R. sylvatica, R. palustris, and a few with R. pipiens (virescens) and Bufo americanus (lentiginosus). No essential differences in behavior between these species were observed. The embryos of R. esculenta and R. palustris seem best adapted for the operation because the dorsal curvature is minimal in those species.

The embryos used in the experiments had to be taken at a stage before the breaking up of the ganglion crest and the migration of the cells between medullary cord and myotomes. Those with barely perceptible tail bud, shortly after closure of the medullary folds, were used for the purpose. At this stage Rana esculenta embryos vary from 2.7 to 3.0 mm. in length, sylvatica embryos from 3.3 to 3.7 mm., palustris from 2.9 to 3.2 mm., and pipiens from 2.4 to 3.0 mm. There were slight differences in the stage used and in the measurements of different strains of the same species at a given stage.

In such embryos the proliferations of the ganglion crest in the head are already well along in their development. In the trunk, on the other hand, the ganglion crest, while visible, lies entirely above the level of the plane dividing the dorsal and ventral halves of the medullary cord, and none of its cells have migrated below the dorsal border of the myotomes (fig. 27, *g.cr*). In the posterior part of the trunk it rests entirely intact upon the roof of the medullary cord.

The experiment consists in removing a thin strip from the back of the embryo (fig. 12, A), either with a very small scalpel or better with a pair of iridectomy scissors. In addition to the ganglion crest, the dorsal half of the medullary cord, a small band of epidermis including the rudiment of the dorsal fin fold, and perhaps a small strip from the dorsal border of the muscle plates are removed by the operation. Owing to the fact that they are already well advanced in development and to some extent because of the cranial flexure, the head ganglia are little if at all injured by the operation, and the embryo develops without much disturbance of function. When the cut has been made at the proper level, the medullary canal appears as a distinct groove (fig. 12, B). If the cut has been carried too deeply, then the notochord appears as a solid rod of cells between the muscle segments on each side. If it is not deep enough, then the medullary cord either appears as closed or as too deep a groove. The operation may thus be controlled in the living specimen, but not absolutely, for the boundary between the dorsal and ven-

tral halves of the medullary cord is an imaginary one. Fortunately, however, there is a microscopic criterion by which it may later be judged from sections whether the operation was 'clean' or not. There are two easily recognizable elements known to be derived from the dorsal part of the medullary cord, viz., the spinal ganglia and the Rohon-Beard dorsal giant cells. Presence of either type of cell is therefore sufficient to cast suspicion upon the precision of the operation. In fact, the three elements, spinal ganglion cells, dorsal cells, and sheath cells, are found to vary concomitantly.

In order to prevent possible regeneration of the dorsal half of the cord, it was at first thought best in each experi-

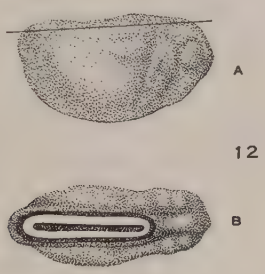

Fig. 12 Rana esculenta embryo in the stage at which the ganglion crest was removed in the experiments. A, profile view; line indicates incision. B, view from above showing the open medullary tube. × 9.5.

ment to unite back to back by Born's method two embryos operated upon in the manner just described. Owing to the fact that the wound surface is very narrow, the complete union of the two embryos often failed. It became apparent afterwards, however, that such a procedure was unnecessary.

If the two components fail to heal together, the wound made in cutting off the strip nevertheless heals rapidly, and the embryos continue their development normally except for the defects caused directly by the wound (fig. 19). Little or no regeneration of any of the parts removed takes place. The cases in which the two components heal evenly together (figs. 13, 15, 16, 17, 18) are more suitable for study because the tails grow out straight, the presence of a fin fold on each

border of the double axial musculature making the tension of the parts correspond more nearly to the normal. In many of the single embryos and in those where only partial fusion of the two components took place the tails were considerably crumpled. Most of the latter cases, however, developed a more or less extensive portion of the dorsal fin in the distal part of the tail (fig. 19).

Microscopic examination has shown the spinal cord to be more complete in the tail, beginning near the base, than in the trunk. This is not surprising, since the extreme distal part of the medullary tube and the tail fin develop, even when everything is removed from the dorsal ridge of the embryo down to the notochord. These structures are formed, respectively, from the walls of the neurenteric canal and the covering ectoderm, where there is a growing point which remains after operation.

Recently a few experiments in removing the ganglion crest have been made with Amblystoma punctatum, but with a very peculiar result, which needs further investigation. On account of the marked dorsal curvature, two embryos could not be healed together. When left single, however, it was found that the walls of the medullary tube did not heal over, as in the anuran embryo. Instead, each lip fused with the adjacent epidermis, leaving an open medullary groove, which remained in this condition as long as the embryo lived. Unfortunately, none of these cases survived long enough for the desired purpose.

The behavior of the embryos used in the experiments is clearly modified by the operation. They vary considerably in their locomotor activities, but all of them show ataxia to some extent. Some reacted to mechanical stimuli merely with a slight quiver or single jerk. These were cases in which the incision had been made at too deep a level, with the consequent ablation of some of the motor elements. Others, which swam well, were found not to have had all of their sensory elements removed. Several cases, however, which subsequent examination showed to have very defective spinal ganglia or

even none at all, were able to swim fairly well and to take food after the absorption of yolk.

Altogether 100 experiments have been made in this series. Nearly half of these have been examined microscopically, some in serial sections and some by means of total preparations of the abdominal walls. The former were cut for the most part in a plane slightly divergent from the sagittal, so as to show the motor roots for as long a stretch as possible after they issue from the medullary cord. The abdominal wall preparations were of especial use in showing the extreme peripheral distribution of the motor nerves.

In the description of the normal development of the motor nerves it was pointed out that the nerve fibers are, in the younger stages, sometimes quite free from cells, and that although they grow out through a rather concentrated mass of cells, formed by the rudiments of the spinal ganglia and the sclerotomes, there are no definite relations between these cells and the nerve fibers. It is, therefore, not to be expected that the earlier stages of development of the motor roots in operated embryos will show any marked deviation from the conditions obtaining in the normal. We may begin, then, with the description of an embryo killed three days after the operation.

Experiment Z. 20, R. esculenta. In this case (fig. 13) the two components healed together very evenly from the neck to the base of the tail. The axis of the tail forks distally and a fin fold, the remnant of the two dorsal fins, is found between the two branches. At the time of preservation the double embryo could actually swim a little, though not so well as the control (fig. 14). Sections show that the medullary cord forks in the region of the twentieth segment and runs to the tip of each branch of the tail axis. The spinal canal is considerably dilated in the distal part of the single portion and numerous cells lie within the cavity, both there and in the branches. In the head region a number of these cells send off long naked nerve fibers which run in the ventricular cavity.

In the walls of the spinal cord there are numerous neuroblasts. The dorsal (giant) cells of Rohon are, as would be expected, wanting. However, distal to the point of birfurcation there are a few of these large cells situated on the dorsal side of both of the branches of the cord. There are no spinal ganglia whatever in either component, and no sporadic ganglion cells are visible, although it is barely possible that, owing to the early age of the specimen, single ganglion cells might be present and overlooked. All circumstances, however, justify the assumption that the operation was a clean one, i.e., everything was removed that was intended to be removed except possibly at one point, and not too much was taken away from any region.

Motor roots were found in connection with nearly every segment of the body. The inability to find them in some of the proximal segments of the trunk is probably due to the extremely unfavorable plane of section in that region. Throughout the distal part of the trunk and the proximal part of the tail, in fact quite out to the point where the medullary cord forks (region of the twentieth somite), the motor roots in many instances (cf. fig. 34, drawn from a specimen two days older) can be followed for a considerable distance from the cord. With two possible exceptions, these nerves are naked. They consist of bundles of still finer fibrillae, but the individual fibrillae are not nearly so plain as in older specimens. The individual nerves take the same course as in normal embryos, those farthest out on the tail running a considerable distance in a longitudinal direction. The possible exceptions just referred to are the last two nerves given off the cord just before its bifurcation. On each one of them there is a single cell, pretty closely applied, which might possibly be interpreted as a sheath cell though probably mesenchyme. Moreover, this is close to the region where a few Rohon cells are to be found in the cord, and it is possible the dorsal part of the cord was not altogether excised in this region. The motor roots which are cut most favorably are those of the thirteenth, fourteenth, and fifteenth somites of one of the components.

Experiment Z. 23, R. esculenta. This specimen (fig. 15) was killed four days after the operation. At that time the two components viewed from the surface were very evenly healed together except for an epithelial scar on one side. Viewed as a transparent object, the two notochords were seen to be perfectly distinct in the tail, and the medullary cord between the two appeared single. The double organism reacted readily to light mechanical stimuli, i.e., needle point

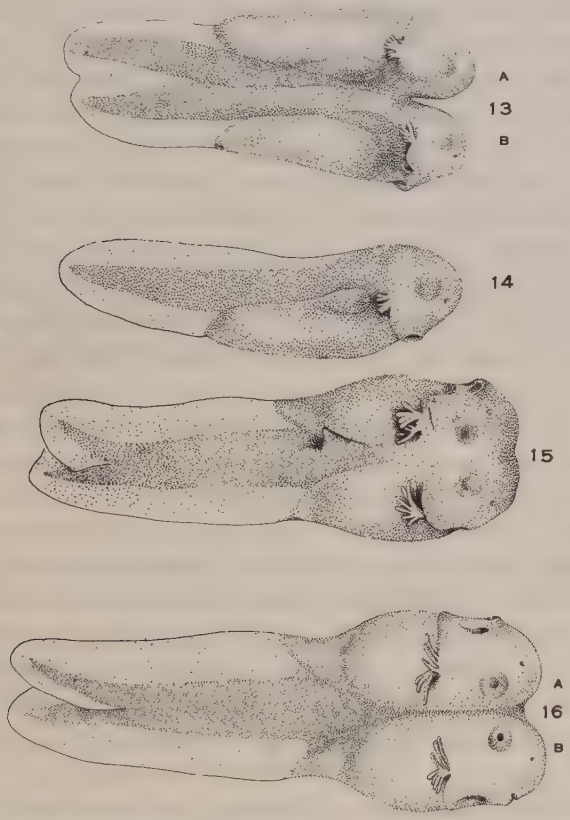

Fig. 13 Double embryo united after removal of dorsal strip, including ganglion crest, three days after operation. Experiment Z. 20 (R. esculenta). × 10.

Fig. 14 Normal control embryo (R. esculenta). × 10.

Fig. 15 Similar case four days after operation. Experiment Z. 23 (R. esculenta). × 10.

Fig. 16 Similar case five days after operation. Experiment Z. 19 (R. esculenta). × 10.

applied to the head, and could swim a little. As will be seen below, the operation in this case was not so exact as desired, and yet the specimen is very instructive in some respects.

The series of sections shows that the relations of the central nervous system of the two components are more complicated than supposed. In the head the two brains are united. From here the cord is continued backward to the tail of one of the components. Joined to this in the region of the seventh or eighth myotome is a piece of medullary cord which belongs to the other component. It extends anteriorly to the region of the third myotome, whence it sends further forward a bundle of fibers, the exact distribution of which could not be determined. In the distal half of the tail the medullary cord, which is continuous from the brain through one of the components, bifurcates and one branch follows each notochord to the tip of the tail axis. In the anterior part of the first component the operation was evidently not exact, for in connection with the nerves in this region there are small ganglia and sheath cells, at least on one side of the body. On the other side, owing to the unfavorable plane of section, the relation of these nerves cannot be satisfactorily made out.

In the other component the nerves of the anterior part of the trunk are derived from the branch cord mentioned above and, as far as can be made out, are devoid of spinal ganglia and sheath cells. The nerves of this component, from the seventh to the twelfth segments, inclusive, bear small spinal ganglia and a varying number of sheath cells. The thirteenth nerve and the following ones lack ganglia and sheath cells. Some of these nerves afford beautiful instances of sheathless fibers and they may be followed far ventrally in the musculature (cf. figs. 37 and 38, taken from an older specimen). In the region about the twenty-first segment isolated sheath cells again appear, though also from here on many nerve filaments are entirely lacking in these elements. The latter nerves evidently proceed from the giant cells of Rohon, found in this part of the cord. A few spinal ganglion cells are also present in this region.

This case is of importance in showing clearly the concomitance of the spinal ganglion cells and the sheath cells. In regions where the former are present we find the latter; where ganglia are absent, the sheath cells are also absent except in the distal parts of the tail where, however, Rohon-Beard cells are present.

Experiment Z. 19, R. esculenta. In this experiment embryos were used that were slightly younger than usual. The two components healed very evenly together with little or no scar. The tail axis is straight and only the distal third of the appendage is bifurcated. The specimen (fig. 16) was preserved five days after operation. The larva at that time could not swim well, but it did react to stimuli, applied to the head, by contraction of muscles of the trunk and tail.

The sections show that the medullary cords of the two components are well healed together throughout the head, trunk, and proximal part of the tail. Only in the distal part of the tail is the cord bifurcated. The notochords of the two components are entirely separate, the combined medullary cord lying between them.

Throughout the trunk region and at the beginning of the tail there are no spinal ganglia whatever, not even single ganglion cells. The first ganglion present belongs to the nerve running to the fifteenth myotome. From this point on, in the tail, ganglia are present and sheath cells occur on their nerves. In the trunk region and, in fact, wherever the ganglia are absent, the nerves are absolutely bare of sheath cells and may be followed for considerable distances beyond the point where they issue from the cord (fig. 34). The nerves that belong to the region of the posterior extremity show very clearly, and the more outlying stretches running to the primary abdominal muscle afford beautiful pictures of sheathless fibers (cf. fig. 35 taken from another specimen). The large nerve trunks of the anterior segments are also very striking in comparison with the cellular nerves of this region in normal embryos.

Dorsal cells are found in the cord from the fifteenth myotome on for some distance in the tail. In front of this point

two such cells in the region of the twelfth and thirteenth segments were observed. Otherwise there are none, unless perhaps a row of large cells of doubtful nature in the region of the first myotomes of one side are to be considered as such.

Experiment Z. 8, R. esculenta. This individual (fig. 17) was kept alive for six days after the operation. The healing of the wound was good, but even in the living specimen it could be seen that there was but one notochord in the tail and that the medullary cord did not extend to that region. Sections show that the nervous system of the two components is separate except in the head, where there is fusion for a short distance. The spinal cords of the two individuals are separate, and each terminates in the posterior part of the trunk. From the end of each a bundle of medullary fibers runs for some distance through the mesenchyme. The notochords of the two components fuse opposite the seventeenth myotome and a single structure runs to near the tip of the tail where it bifurcates. The medullary cord is lacking from the point of fusion of the notochords to their distal bifurcation, from which point a short stretch (0.23 mm.) of medullary cord, consisting only of epithelial cells, runs to the tip of the tail axis.

In the case of one of the components the cut must have been made in a slightly oblique direction, for in the anterior part of the trunk spinal ganglia are developed. In the tail the whole cord was cut out, while in the posterior region of the trunk only the ventral part was left. On both sides of the body the ganglia belonging to the fourth, fifth, and sixth segments are of fair size, though not so large as in normal specimens. The seventh segment has a small ganglion; on one side it consists of but two or three cells; on the other side it is a little larger. The nerves of these segments are all provided with sheath cells. In the eighth segment the motor roots are distinct, but no ganglion cells are present. There are no sheath cells in connection with this nerve. The motor roots of the ninth and tenth segments are perfectly free from cells. They are well developed and in one case at least may

be followed to the primary abdominal muscle. The distribution of the Rohon-Beard giant cells is very instructive. In the cord of the anterior part of the trunk there are a considerable number of these cells, which are normally differentiated. The last one visible is on the border between the seventh and eight segments. We thus see that in this specimen

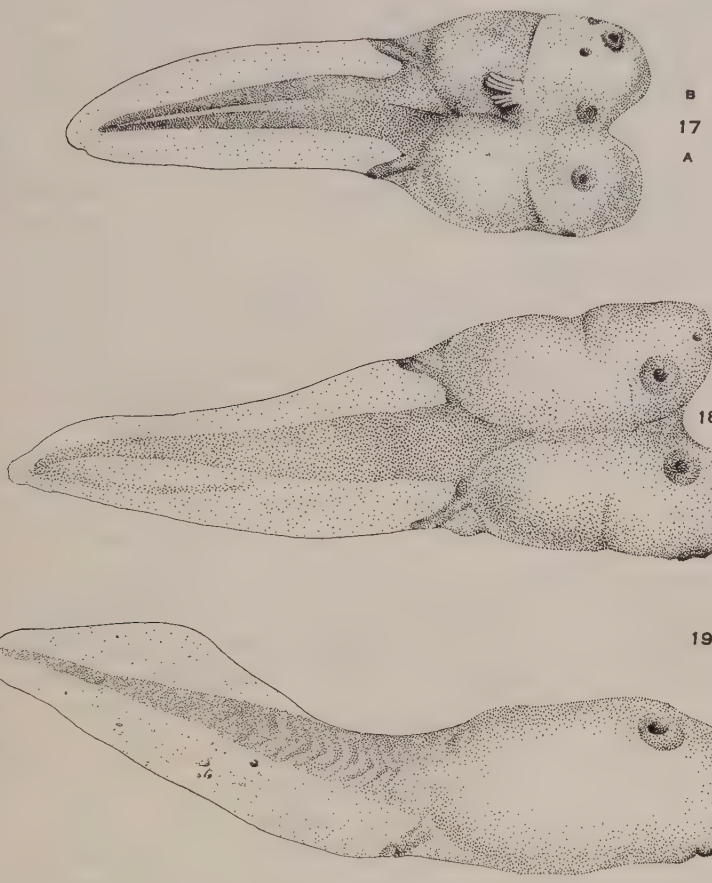

Fig. 17 Case similar to the one shown in figure 13, six days after operation. Experiment Z. 8. (R. esculenta). × 10.

Fig. 18 Similar case eight days after operation. Experiment Z. 24. (R. esculenta). × 10.

Fig. 19 Single larva from which ganglion crest had been removed as in previous cases. Experiment Z. 56 (R. palustris). × 8.7.

there is again a close parallel in the distribution of the three elements—the spinal ganglion cells and the dorsal giant cells on the one hand and the sheath cells of Schwann on the other.

In the other component the conditions are quite different, the only similarity being that in the two cases the medullary cord was entirely obliterated distal to the region of the tenth myotome. In contrast to the first component spinal ganglia are here entirely absent. The motor roots are well developed, and those on one side, which are more favorably cut, show no sheath cells. On the other side the nerves are not cut so favorably, and in some cases the relations of the mesenchyme are such that perfectly clear pictures are not to be had. On the nerve to the fourth myotome there is a spindle-shaped cell which may possibly be a sheath cell, otherwise there are no cells of this character in this component. Nor are dorsal cells present in this component except one in the region of the fourth segment. The clearest nerves are those of the segments in the anterior part of the trunk. The contrast between these and the corresponding nerves in normal specimens, which are extremely cellular, is very striking.

Experiment Z. 65, R. palustris. This case is that of a single embryo not united with another. It was preserved seven days after operation, at which time it had not developed so far as some others of the same age. Sections show considerable histolysis in the central nervous system and some vacuolization of the musculature. In the anterior part of the trunk on the left side several small ganglia are present, and the nerves of this region have a small number of sheath cells. This specimen is particularly interesting in showing the ventral portions of the posterior trunk nerves—ninth and tenth. These are seen on both sides in long stretches with ramifications (fig. 35). The neurofibrillae are distinct and no sheath cells whatever are present. Normal nerves from the same region are shown in figure 36, drawn from a normal Rana sylvatica embryo, the control in another experiment (Z. 77).

Experiment Z. 24, R. esculenta. This specimen (fig. 18) was allowed to live for eight days after the operation. The

healing together of the two components was good. The tail was straight, with irregularities only at the end. Two notochords with one combined medullary cord between them were observed in the living specimen in the proximal part of the tail. The double larva could swim fairly well and was extremely irritable to mechanical stimuli. Sections show that the notochords fuse in the distal half of the tail and that one notochord and the medullary cord extend to the end.

The medullary cord of the two components is healed to form one tube, extending from the head to the tip of the tail. Throughout the whole of the specimen spinal ganglia are absent, with one exception; in one component a small ganglion belonging to the tenth segment is present on one side. The nerve of this segment is one of those running to the hind limb. It is plainly visible between the ventral border of the muscle plates and the base of the limb bud, where it has a few sheath cells. On the neighboring nerve, the ninth, are likewise two sheath cells situated below the ventral border of the muscle plates. The rest of the nerves in the trunk and along the proximal part of the tail as far as the eighteenth segment are free from sheath cells. Those belonging to the region of the posterior extremity on the opposite side of this component and the corresponding nerves in the other component may be followed between and below the myotomes to the base of the limb and the primary abdominal muscle. Also ventrally, between the myotomes in the proximal part of the tail, naked bundles of fibrillae may be readily seen. The nerves of these regions, as they leave the cord, are also clear bundles of fibrillae without a trace of sheath cells. Near the point of exit of some of the nerves in the anterior part of the trunk mesenchyme cells with round nuclei are collected, but nowhere are there spindle-shaped cells lying on the nerve fibers, and the peripheral stretches of these nerves are strikingly free from cells, in great contrast to the corresponding nerves in normal specimens.

The distribution of the Rohon-Beard cells is interesting. Throughout the trunk region and also in the proximal part

of the tail a few of these cells were found. In the sixth segment there is one, in the seventh two, and in the tenth, twelfth, fifteenth, and seventeenth segments, one each. Beginning with the eighteenth segment, which is the one where the peripheral nerves begin to show sheath cells, there is a chain of these cells, which extends far out along the tail. Here there are also a number of groups of sporadic ganglion cells outside the cord.

Experiment Z.77, R. sylvatica. This case was kept nine days after the operation. The nerves in the posterior part of the trunk and the base of the tail are favorably cut. Figure 32 shows motor (eleventh spinal) nerves arising from the fused medullary cord and running to each of the two components. For comparison, the arrangement of a segmental nerve, with spinal ganglion and both roots covered by numerous sheath cells in a normal control larva, is shown in figure 31. This specimen is also of interest in showing correlation between the presence of spinal ganglia and the occurrence of sheath cells on peripheral nerves. There are many long stretches of nerves without sheath cells median to the myotomes, but in one of the components one of the nerves has a number of such cells. This branch, however, is found to be connected with the only nerve (twelfth spinal) in the whole specimen having a spinal ganglion—a small structure shown in figure 33. The root has only one sheath cell. One other nerve has a single sheath cell just where it emerges from the cord—within the cord are two cells near the point of exit. This probably indicates the beginning of the migration of cells along the ventral roots, which becomes more active in later stages.

Portions of the nerve plexus medial to the muscle plates are shown in figures 37 and 38. These consist of anastomosing bundles of fibrillae without sheath cells. In the control larva of the same age there is a marked confrast, for numerous sheath cells are present (fig. 39). The same contrast is shown in the nerves in the region of the hind limb. (Cf. fig. 36, taken from the same normal control, with fig. 35 drawn from

another specimen, Z. 65, from which the ganglion crest had been removed.)

The next two cases are interesting in showing that some time after the absorption of the yolk, the motor nerves acquire sheath cells, even though absence of spinal ganglia indicates that the ganglion crest was entire obliterated.

Experiment Z. 56, R. palustris. This case, a single individual, i.e., not united to a mate, was preserved sixteen days after operation (fig. 19). It could swim fairly well, and when the tail was removed immediately before preservation the trunk and tail stump responded vigorously to stimuli. Sagittal sections show that there are no spinal ganglia present. The motor nerves throughout the anterior part of the trunk have sheath cells, which are more numerous anteriorly. There are a few as far back as the sixth or seventh nerve. Behind this the motor nerves have none whatever. The sections are favorably cut for study, and naked axis cylinders may be followed to the posterior limb bud. In the last nerves which have sheath cells the latter are found only near the medullary cord, indicating that they arise in the cord and wander out.

Experiment Z. 36, R. sylvatica. This case also was a single individual. The larva, two days after the operation, could swim fairly well for its age, though not so well as a normal one. This condition continued, and as the specimen could feed, it was kept alive for thirty days, during which time it grew to a length of 17 mm. It remained, however, considerably smaller than the control. The caudal fin was present in the distal part of the tail. The swimming movements were abnormal throughout the life of this individual, but with the tail cut off near the trunk, stimuli applied to head or body resulted in vigorous reactions.

The specimen was cut into sagittal sections. In the trunk region there are two small ganglia on the left in connection with the first and second spinal nerves, respectively, and three on the right connected with the third, fourth, and sixth. The motor roots of the spinal nerves are all well developed, but,

except where ganglia are present, there are no dorsal roots. The motor nerve trunks are large and well supplied with sheath cells, and some of the fibers are medullated. There is nothing in the specimen to indicate where the sheath cells have originated. Probably, however, they wandered out from the cord along the ventral roots of the nerves.

In order to examine the extreme peripheral course of the sheathless nerves, some of the specimens were dissected to show the abdominal muscles (fig. 20). After placing the preserved specimens in water for a short time to render them less brittle, the epidermis was stripped off and the whole of the abdominal wall, including the ventral border of the myotomes, was cut out and mounted. Dissections of this kind can be readily made after preservation in corrosive sublimate, but these are histologically not so satisfactory as specimens fixed and stained by O. Schultze's ('04 c) haematoxylin method. Unfortunately, however, material treated by the latter method is very brittle and can scarcely be dissected if kept any length of time in alcohol. According to this method, the larvae are stained in toto in haematoxylin directly after fixation in Tellyesniczky's fluid. The preparations are mounted in potassium acetate, which, owing to its low index of refraction, brings out many finer details that are completely lost in dammar or balsam. Their durability is, however, not great, and all which I have prepared in this way have in time become worthless. Normal specimens treated in the same way were also prepared for comparison. These preparations are so thin that they may be examined even with the oil immersion.

As is well known, the primary abdominal muscle begins as a narrow band, which leaves the myotomes at the base of the tail, runs anteriorly and ventrally, skirting the hind limb bud, and spreads out in the abdominal wall into a thin sheet on each side of the mid-line, extending anteriorly to the branchial region (fig. 20 a). In the abdominal portion there is a considerable gap left between the ventral border of the axial musculature and this muscle.

In normal specimens the nerves may be traced from the border of the myotomes across this space and into the abdominal musculature (figs. 20, b and 40). The motor rami (*Mot.N*) enter the latter at the inscriptiones tendineae, which they follow on the inner surface to the medial or ventral border, giving off twigs to the muscle fibers on each side of the septum. Before reaching the abdominal muscle the nerves

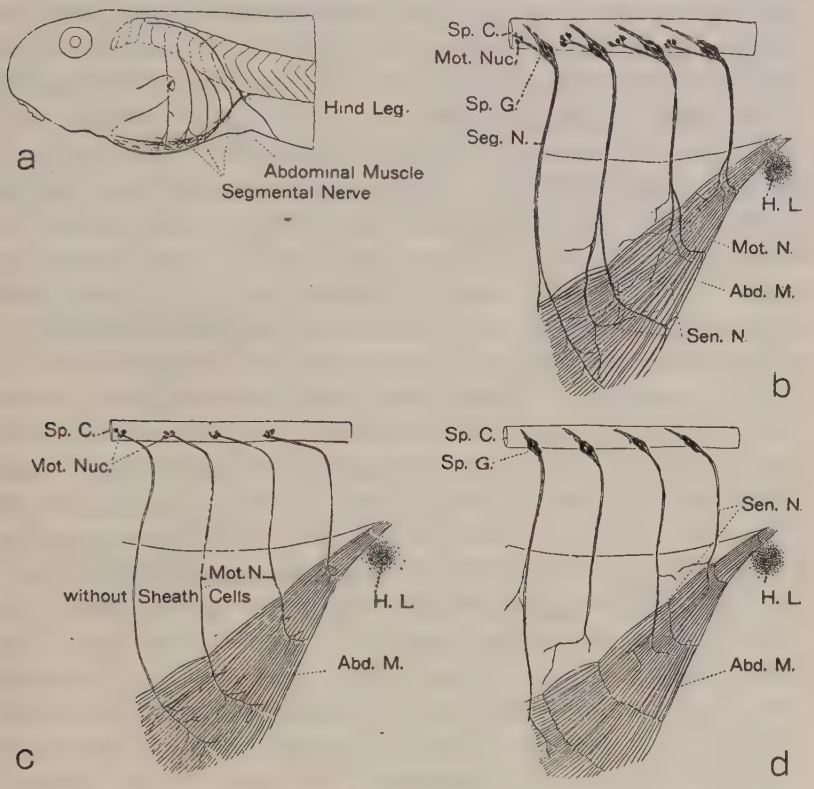

Fig. 20 Diagrammatic views of the nerves in the abdominal walls of tadpole: a, body of larva showing general arrangement of nerves; b, detailed arrangement in normal larva; c, arrangement in larva from which ganglion crest has been removed, only the motor nerves showing; d, arrangement in larva from which the ventral half of the spinal cord had been removed, showing only sensory nerves. *Abd.M.*, primary abdominal muscles; *H.L.*, hind limb; *Mot.N.*, motor nerve; *Mot.Nuc.*, motor nucleus; *Seg.N.*, segmental nerve; *Sen.N.*, sensory nerve; *Sp.C.*, spinal cord; *Sp.G.*, spinal ganglion.

give off sensory branches (*Sen.N*), which run outside of the musculature and form anastomoses with each other under the epidermis. No anastomoses between adjacent motor rami have been found in the stages used. The abdominal nerves, both motor and sensory, are provided with numerous sheath cells (fig. 40).

Quite a different picture is shown by the larvae from which the ganglion crest has been removed (figs. 20, c and 41). Here the nerves appear as fine threads composed of primitive fibrillae and entirely devoid of cells (fig. 41) except in isolated cases to be mentioned below. The course of the nerves is the same. They run from the ventral border of the myotomes across the space in which no muscle is present to the ventral muscle which they enter, as in normal cases, at the inscriptiones tendineae, and give off branches to the muscle fibers on each side. The nerves finally terminate at the medial border of the muscle. The nerves to the skin, which correspond to those connected with the spinal ganglia in normal cases, are absent in the specimens operated upon.

Twenty-eight individuals have been examined in this way. As was to have been expected, many of them proved to be negative, i.e., no nerves, either sensory or motor, were found. These were presumably individuals in which the incision had been made too low. The effect of this would be noticed first in the more outlying regions of nerve distribution. More than half of the specimens showed motor nerve fibers, with total absence of sensory nerves. In nearly all cases they were entirely without sheath cells; only in three were any such cells found and then merely in limited numbers on single nerves, other segmental nerves being entirely without them. These latter cases are presumably like those which showed a few sheath cells in sections—cases in which the incision was a little too high. Sections of the axial part of the larva would probably have revealed rudimentary spinal ganglia or at least Rohon-Beard cells.

The following tabulation summarizes the results of the findings in this series of preparations, each side of each individual

being counted separately. A few cases in which the preparations were badly damaged in preparation are not counted.

Total number serviceable preparations,	49 cases
No nerves present at all,	20 cases
One or more sheathless nerves present,	27 cases
Both sensory and motor nerves present,	2 cases
Small number of sheath cells present on one segmental nerve, other nerves being sheathless,	3 cases

b. The effect of removing the ganglion crest and the placodes of the head. As is well known, the development of the cranial nerves is more complicated than that of the spinal. The sensory components are derived from two sources, the ganglion crest and the placodes, principally the latter, and the motor nerves, like those of the trunk, are connected with cells lying within the central nervous system. There is a possibility that sheath cells may be derived from all three of these sources.

Removal of either the ganglion crest or the placodes from one side of the head of young embryos was found to have no effect on the composition of the motor nerves, sheath cells being found after such operations, as in normal specimens. These experiments need not be considered further in this place, since the subject has recently been exhaustively treated by Stone ('22).

To test the rôle of the sheath cells in the formation of the cranial nerves, as was done with the spinal nerves, it consequently seemed necessary to remove both the placodes and the ganglion crest. This was done in a small number of cases, with some variation in the procedure, but the results in the main were disappointing, only two cases being at all conclusive.

Since the ganglion crest of the head in the anuran embryo develops very precociously, it is preferable to use for the operation much earlier stages than those in which the ganglion crest of the trunk was removed. Thus the best case was one in which the operation was done while the medullary folds were wide open.

Experiment G.S.O. 5. An embryo of R. palustris with wide open medullary folds was used in this case. The skin was carefully removed from one side of the front part of the embryo, including all that would later have covered that side of the head below the dorsal mid-line, and extending from the anterior end of the head back beyond the vagus region. Then the proliferations of the ganglion crest were cut off, and finally a large flap of skin from the abdominal wall was taken from another embryo in the same stage and trans-

Fig. 21 Embryo from which the ganglion crest of the head and the placodes had been removed before closure of the medullary folds. Specimen preserved four days after operation. Experiment G.S.O. 5 (R. palustris). × 10.

Fig. 22 Similar case, except that the operation was done after closure of medullary folds and the dorsal part of the brain, in addition to ganglion crest and placodes, was removed. Specimen preserved four days after operation. Experiment G.S.O. 7 (R. palustris). × 10.

planted to the first. In this way the whole ganglion crest of the head, together with the rudiments of all the placodes, was removed.

The wound healed fairly well and the next day was open at only one small point. An epithelial excrescence was formed, however, out of the transplanted skin on the side of the head. The embryo (fig. 21) was preserved four days after operation and was cut into serial sections in a plane slightly inclined to the sagittal.

The operation naturally resulted in distortion of the head. This was manifested in the position of the mouth, which opened to the right, the side of the operation. The sections showed total absence of the visceral arches and the auditory vesicle on this side. The nasal pit is developed, as also the eye, but the latter is imperfect, the optic cup not being normally invaginated and the pigment layer of the retina not differentiated. The lens is absent.

The cranial nerves, which concern us especially, show the following relations. The ganglia are all absent as such, although in the region of the trigemino-facial complex and of the vagus there are a few loose ganglion cells. Owing to the absence of many important landmarks, it is not possible to identify exactly all of the peripheral nerves. There are, however, three distinct groups of fibers, which, except for a very few cells closely applied to them near their origin, consist of naked axones in which fibrillation is very distinct (fig. 42).

The first of these bundles of fibers represents, no doubt, the oculomotor nerve (*n.oc.mot*). It arises by two bundles from the floor of the midbrain and proceeds in a ventro-antero-lateral direction to a group of cells (*mes*) lying dorsal to the inner part of the eye. Its ramifications end among the mesodermal cells. There are no Schwann sheath cells on any part of this nerve.

Further towards the tail the second group of fibers is found, representing the trigeminus, facial, and perhaps the abducens, though it is not possible to identify individual roots or branches. This group arises as three roots, which run together shortly after leaving the medullary cord. Around these roots and at their junction there are a number of cells, some of which are undoubtedly sheath cells, while others are ganglion cells. The origin of the root fibers from pear-shaped nerve cells situated within the medullary cord is very distinctly shown. The peripheral branches of the nerve trunk are entirely without sheath cells. Some of these are also shown in figure 42 (*n. trigem*). They extend for a considerable distance as bare axis cylinders, with distinct fibrillation,

but their mode of ending cannot be made out. The twigs run in a ventro-anterior direction, one of them forming an anastomosis with the first nerve above described.

The third group of fibers, consisting of the glossopharyngeus and vagus arises like the second. There are, however, five distinct bundles of root fibers. These are partially surrounded by sheath cells with a small cluster of ganglion cells and several single ones in the vicinity. These fibers pass ventrally and become lost in the mesenchyme not far from the pericardium.

Behind the vagus the first spinal nerve is to be found. Although there is a rudimentary ganglion in the segment belonging to this nerve, the latter is entirely without sheath cells.

On the whole, this case may be accepted as corroborative of the experiments in which the ganglion crest of the trunk was excised. In the anterior part of the head, i.e., in the region of the third cranial nerve, the removal of the sources of the sheath cells was evidently complete, while in the trigemino-facial and vagal regions it was not quite so. The nerves, however, are far less cellular than in normal specimens, and it is only the proximal stretches that show them. That the operation was not perfectly clean in these regions is shown by the fact that a small number of ganglion cells remain there. The sheath cells may have had a common origin with the ganglion cells, but the sections suggest that some of the sheath cells may be migrating directly from the medullary cord.

Experiment G.S.O. 7. The operation in this case was more radical than that in the preceding. A thin strip was cut off from the crest of the brain, as in the operations for removal of the ganglion crest of the trunk region; then the skin, including all the placodes, was taken off the side of the head and the proliferations of the ganglion crest removed. Abdominal ectoderm with mesoderm cells sticking to it was used to cover the wound. The healing was good and the specimen was preserved after four days (fig. 22). There is one large

nerve on the injured side which arises from the medulla. It is about in the region of the facial but the landmarks are insufficient for its identification. It runs ventrally for a considerable distance, finally losing itself in the mesenchyme over the pericardial cavity. With one or two doubtful exceptions it has no sheath cells. The mesenchyme through which it runs is rather dense. No ganglion cells were found on the injured side.

Six other cases have been examined in sections. One individual (G.S.O. 1), preserved eight days after operation, showed a few ganglion cells in the trigemino-facial region and also a few medial to the first myotome in the territory of the vagus. The oculomotor nerve and fibers in the region of the trigemino-facial and vagus are present, but all have some sheath cells. The sections give evidence of outward migration of such cells along the motor roots. In another case (G.S.O. 2), preserved six days after operation, only the vagus nerve could be found, and it had some sheath cells. Two other cases (G.S.O. 3 and G.S.O. 6), in which the wound was left uncovered after excision and skin from the surrounding region allowed to grow over it, showed considerable regeneration of the trigemino-facial and vagus complexes. Large ganglia and normal-looking nerves were present in both of these cases. One case (G.S.O. 4) showed marked histolysis of the brain and was altogether inconclusive.

The disadvantages of this experiment, as compared with that of the removal of the ganglion crest of the trunk, are not far to seek. The more radical operation in the former disturbs or destroys important landmarks, modifying the course of the remaining nerves and rendering their identification almost impossible. It is difficult to remove the ganglion crest cleanly, and there is a tendency for it to regenerate from the remaining brain tissue. Sheath cells apparently begin their migration from the medullary cord along the motor roots at an earlier stage than in the trunk. For the above reasons, the few experiments which have been made

have not proved to be very satisfactory, and, taken by themselves, would not be of much importance. Nevertheless, it is believed that they are of some value in confirming the experiments described in the previous section.

2. Experiments in eliminating ganglion cells while retaining the sheath cells

The sheath cells are shown by the foregoing experiments not to be a necessary factor in the formation of the peripheral axones. It nevertheless might be possible that they form some of the axones either normally or vicariously in the absence of ganglion cells. Several experiments have been devised in order to test this possibility by removing the ganglion cells and at the same time retaining the source of the sheath cells. There are two groups of these experiments. In the first the attempt was made to remove the motor cells before any nerve fibers were present; the ventral half of the medullary cord containing these cells was cut out, leaving the dorsal half of the cord and the ganglion crest intact. In the second certain nerve centers were removed after nerves had begun to grow and a considerable number of sheath cells were scattered along their course. The latter experiments fall into two sets. In one the whole medullary cord was removed by cutting between it and the notochord after the spinal ganglia had reached their definitive position. This removes the whole of the motor centers, but only the dorsal part of the spinal ganglia, and leaves intact the ventral portion of the ganglia, which extend along the side of the notochord. In the other set the vagus ganglion was removed after the r. lateralis had grown out. The effect upon this nerve was then studied.

a. Effect of removal of the ventral half of the medullary cord. In performing this operation it is necessary first to cut a narrow strip from the dorsal ridge of the embryo, including the dorsal half of the cord. This is done as in the operation for the removal of the ganglion crest, except that the strip

is left attached at its anterior end. By another cut the ventral half of the medullary cord is excised, bringing the notocord into view. The dorsal strip is then healed back in place. The object of this experiment was to ascertain whether cells from the ganglion crest, in the absence of the motor ganglion cells, would form the purely motor rami of the spinal nerves.

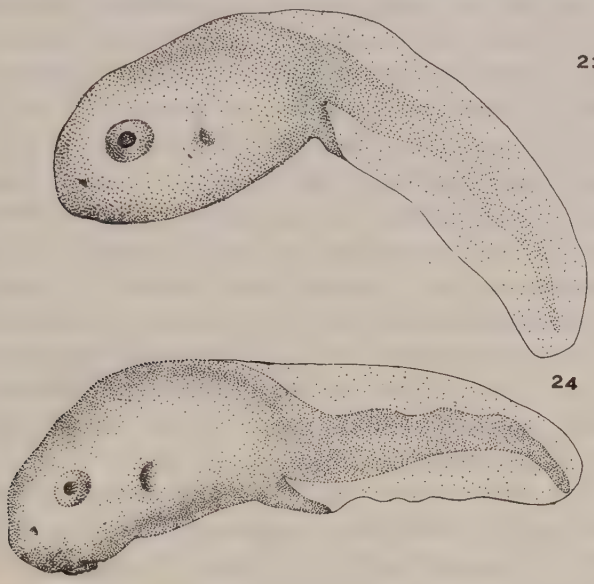

Fig. 23 Individual from which the ventral half of the medullary cord has been removed. Specimen preserved eleven days after operation. Experiment Uj. 5 (R. palustris). × 10.

Fig. 24 Similar case twelve days after operation. Experiment Uj. 2 (R. palustris). × 10.

As pointed out in the previous section, the sensory and motor rami in the abdominal walls can be readily distinguished.

There are difficulties in carrying out the experiment, because it is not possible to heal back the thin dorsal strip without producing some irregularities in the path of the outgrowing nerves. All of these cases show some external deformity (figs. 23 and 24). In addition it seems practically impossible to remove all of the motor elements by this regional separation, unless the first incision is made at a high

level, in which case it usually happens that the small amount of nervous material left in the dorsal strip is insufficient to form a continuous cord. The presence of motor nerves is shown functionally by the fact that the larvae a few days after the operation give slight motor reactions. They cannot execute coordinated movements but frequently show tremor in some part of the axial musculature. That this cannot be taken as evidence for the formation of nerve fibers by the sheath cells is shown by the examination of sections, which reveal the presence of a few motor nerve cells in the cord together with motor roots proceeding from them. The motor fibers run to those parts of the axial musculature lying close to the cord, and only exceptionally are they found in the more outlying regions of the abdominal walls. These circumstances render great exactitude impossible in this type of experiment. Besides, the evidence that the experiments give proves to be of a negative character. In spite of these limitations, it is believed that they afford an important addition to the evidence given in the previous section in favor of the neuroblastic origin of the nerve fiber.

The total number of experiments in this series is twenty-nine, sixteen of which were made with Rana palustris embryos and thirteen with R. sylvatica. No specific difference between the two was detected in respect to the outcome of these particular experiments. Fourteen of the specimens were examined as to the nerve distribution in the abdominal walls. They were prepared in a somewhat different manner from that described in the previous section, because it was necessary to preserve intact the entire distribution of the cutaneous nerves. Consequently the whole abdominal wall, including ectoderm, had to be mounted. On this account it was necessary to stain the specimens less densely than is done by the Schultze method. Haematoxylin and Congo red were therefore used. Such specimens, while less clear than the others, are adequate if studied carefully under high magnification. Only one case was inconclusive on account of poor preparation. In two of these cases the remainder of the

specimen, after removal of the abdominal walls, was cut into sections for study of the central connections of the spinal nerves. Four other specimens were studied only in sections. The above are considered to be a fair sample of the whole material, and it is not thought that an examination of the remaining cases would yield different results.

The thirteen abdominal wall preparations (fig. 20, d) may be counted as twenty-six cases, since the two sides are independent of one another as far as innervation is concerned. All nerves, both sensory and motor, were lacking in five cases; motor nerves were absent, but sensory present in seventeen cases; both sensory and motor nerves were present in four cases, but all of the latter showed motor nerves only occasionally, i.e., in one or more segments; in other segments sensory nerves alone were present. The sensory nerves (*Sen.N*) form a plexus underneath the epidermis, reaching to the level of the primary abdominal muscle. Histories of typical cases are given in the following.

Experiment Uj. 16. Embryo of R. sylvatica. The first incision through the medullary cord was well placed, and then the notochord was laid bare from the hindbrain region to the neurenteric canal; the wound healed well. Five days after the operation contraction of proximal myotomes of tail was seen to follow mechanical stimulation of the flank. This may possibly have been due simply to jarring. Otherwise no movement was noticed. Larva shows some kyphosis, otherwise healthy. Ten days after the operation the tail was amputated at the level of the anus. Slight twitching of the axial musculature of the tail stump took place when stimulated sharply. The specimen was then preserved in mercuric chloride and acetic acid.

The abdominal wall preparation was stained lightly in haematoxylin and Congo red. The primary abdominal muscle is less compact than in normal larvae, resembling the muscle in specimens from which the entire spinal cord has been removed. On the right side segmental nerves, from the fifth to the eighth inclusive, are present. The three posterior ones

extend almost to the ventral border of the muscle, lying between the muscle and the skin. No motor abdominal nerves are to be found. On the left side two nerves are present, probably the sixth and the eighth, but neither reaches as far as the abdominal muscle. Motor rami are lacking on this side too.

Sagittal sections of the axial portion of the larva show the spinal cord to be interrupted in the anterior part of the trunk region. A number of well-developed spinal ganglia, somewhat irregular, however, are present on both sides. Two motor roots and a few ventral horn motor cells are found in the region supplying the myotomes at the base of the tail.

Experiment Uj. 2. In this case (R. palustris) the primary incision was made too deep, so that posterior to the dorsal depression the cut lay between notochord and medullary cord. The notochord was then cleaned anteriorly. The result of this was that the whole of the medullary cord from the base of tail on was healed back in place. In the anterior part of the trunk only the dorsal half of the cord was left. Six days after the operation it was noted that, while the control larva could swim well, the one operated upon reacted only by a slight quiver of the head, due to contraction of muscles in front of the region from which the ventral portion of the cord was removed. Five days later, this larva moved its head, as before, and also the tail beyond the anus, the latter on stimulation of head or of abdomen. No movement of the muscles in the middle of the trunk or the base of the tail could be discerned. One day later, or twelve days after the operation, the specimen was preserved (fig. 24). Considerable movement took place when the fixing fluid was applied.

The abdominal wall preparation on the right side near the tail, where the medullary cord was left almost completely intact, shows two stout nerve trunks containing both sensory and motor elements. Anterior to this, sensory but no motor nerves are present. On the left side no motor nerves are found, but the sensory plexus is well developed.

There is an apparent inconsistency here between the physiological and the anatomical findings, since motor nerves were found at the base of the tail, where no movement was observed. The lack of function was probably due to the inadequacy of the nervous connections with the anterior part of the animal.

It is scarcely necessary to describe this material in further detail. The results may be summarized as follows: Removal of the ventral half of the medullary cord just after closure results in the elimination of nearly all of the motor elements. The few that remain give rise to motor nerves which innervate very imperfectly the axial musculature. Occasionally a motor nerve of this origin may reach to the abdominal muscle. The number of motor fibers present is in general dependent upon the level of the incision, which, because of the absence of landmarks and the small size of the embryo, cannot be laid with absolute precision. When too little of the cord is left, its continuity becomes interrupted during the course of development, and only small remnants of it, together with isolated spinal ganglia, remain. In these cases sensory, but no motor, nerves are found. Everything indicates, therefore, that the ventral horn cells are the necessary factor in the development of the peripheral motor nerves, and that the sheath cells, in the absence of ventral horn cells, are unable to form the motor axones.

One or two specimens show cells near the aorta that resemble sympathetic ganglion cells, but the evidence upon the question of their presence and their origin is inconclusive.

b. Effect of removal of the medullary cord after the spinal ganglia have begun their development. The removal of the medullary cord of the trunk region at a time when the spinal ganglia are already in place affects the sensory and motor nerve elements differently. The motor cells located in the cord are eliminated, while a portion of the sensory cells are left intact. The motor nerves, however, had already begun their development at the time of operation, and in this respect the experiment differs from the preceding.

Embryos at a stage shown in figure 5 were chosen for the purpose, because at this stage the spinal ganglia are already in place, and some of their cells reach below the upper level of the notochord and are not removed by the operation; and because the peripheral nerves have at this time probably not reached their extreme ventral termination.

The operation was done under chloretone anesthesia. By a single cut made at the right level it is possible to remove the medullary cord of the trunk without injuring the notochord, and the precision of the experiment can be readily controlled because of the distinctness of the notochord. The specimens, eighteen in number, were preserved from two to five days after the operation. Healing of the wound takes place readily and a large portion of the dorsal fin regenerates (figs. 25 and 26).

Five cases were examined in abdominal wall preparations, and two others were cut into serial sections. Inasmuch as the findings are consistent, with a single exception that may be explained, it has not seemed necessary to extend the minute examination to additional cases.

The two series of sections show that large fragments of spinal ganglia are left and that streams of cells extend ventrally from them between notochord and myotomes. In some places nerve fibers may be found running between the myotomes toward the ventral fin fold.

The youngest of the abdominal wall preparations was taken from an individual (Exp. Y(St.11),1) preserved two days after the operation. On the right side all of the nerves are distinct except the two last trunk nerves (ninth and tenth), in which region the yolk obscures the specimen. The eighth nerve runs to the border of the primary abdominal muscle, while the nerves anterior to this run further between the muscle and the skin. There are anastomoses between the neighboring nerves. No motor nerves are present, though the eighth is not absolutely conclusive in this regard. On the left side all the segmental nerves show. All extend beyond the border of the primary abdominal muscle between the muscle and the skin, but no motor nerves are to be found.

The other cases are similar to this one. Occasionally one of the segmental nerves may be lacking, but this is not surprising, for the mode of operation might sometimes tear out the whole of a given spinal ganglion.

These experiments speak more cogently against the sheath cells having any part in the formation of the axone than do

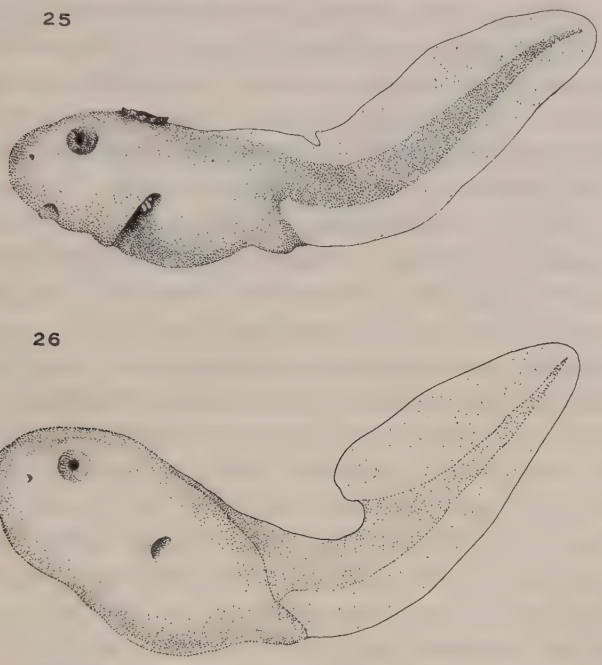

Fig. 25 Larva from which medullary cord was removed after spinal ganglia and sensory nerves were formed. Experiment Y(St. 11).14 (R. palustris), three days after operation. × 10.

Fig. 26 Similar case. Experiment Y(St. 11). 16 (R. palustris), six days after operation. × 10.

the experiments in which only the ventral half of the medullary cord was removed; for in the former the motor nerves had already begun their development, and yet the sheath cells already present on the nerve trunks at the time the connection with the motor centers in the spinal cord was severed do not form the motor rami running to the primary abdominal muscle.

c. Effect of removal of the vagus ganglion on the r. lateralis vagi.[31] The experiments just described show the inability of the sheath cells even to continue the formation of the axone after the first portions of it are laid down. Those taken u in this section, while pointing in the same direction, are not i themselves conclusive. They are reported here principally because of the promise shown by the method used. They were originally designed to bridge the gap in our knowledge of those phases of development which lie between ontogeny and regeneration. This is all the more important, because both ontogenetic and regenerative processes have been intensively studied in relation to our general concepts of the structure of the nervous system, and both the outgrowth theory and the cell-chain theory have found support in such studies.

The vagus ganglion was removed from embryos and larvae of R. palustris in three different stages of development. The youngest were embryos a little over 6 mm. in length, in which the external gills were just beginning to sprout. In the second stage the length was a little over 8 mm. and the external gills had reached the height of their development. In the third the yolk was completely absorbed and the larvae beginning to feed. A fourth series of experiments with older larvae having medullated fibers in the r. lateralis was projected but not carried out. The vagus was selected for this purpose because of the especially favorable relations of the r. lateralis. It is a long nerve, with a superficial course, and it runs independently without forming anastomoses with the general cutaneous nerves that cross its path or with any other nerves. Preparations of the skin from the whole side of the body of the larva are easily made and the nerve with its final ramifications viewed as a whole. The ganglion from which it arises is easy of access and readily removed at any stage without serious injury to the embryo or larva.

[31] This work was originally undertaken in collaboration with Dr. Laurence Selling, but it suffered interruption on account of other demands on our time. It can be reported now only in brief because of its incompleteness.

The first series of experiments was done with embryos in which the lateral-line rudiment had extended, at the time of operation, to the fifteenth or sometimes as far as the seventeenth somite, which is a little beyond the level of the anus. Considerable variation is shown in the extent of the rudiment at this stage, and even on the two sides of the same embryo there may be a difference of more than two somites. There are no differentiated or segregated sense organs at this stage, but mitoses are frequent. Lateralis nerve fibers and Schwann cells extend to within one or two segments of the tip of the sensory rudiment. The excision of the ganglion leaves, therefore, a stretch of the sensory rudiment, extending the whole length of the trunk, and this includes sensory cells, sheath cells, and peripheral nerve fibers, but no ganglion cells or neuroblasts.

The results of this experiment may be stated in a word. After the removal of the vagus ganglion not only does further differentiation of the axones cease, but the nerve structures (axones and Schwann cells), which were present at the time of operation, degenerate completely, no traces of them being visible after five days. They failed to reappear, but the lateral-line sense organs persisted for a considerable time. Specimens preserved two days after excision of the ganglion still show axones and sheath cells in stages of degeneration.[32]

In the embryos used in the second series of experiments the lateral-line rudiment had grown well out on the tail at the time of operation, certainly as far as the thirtieth somite. Axones and sheath cells are more numerous than in the previous stage, and the individual sense organs are segregated, but are not yet much differentiated. Degeneration of the r. lateralis began early, almost immediately after excision of the ganglion, as in the previous series, but proceeded much more slowly. After operations at this stage there is a marked activity in the sheath cells of the injured nerve. Processes from these often leave the nerve trunk and either end freely

[32] Cf. Harrison ('03, pp. 73–74).

a short distance from it or form anastomoses with the network of cutaneous nerves which cross the path of the r. lateralis. Whether the general cutaneous nerves are active in forming these connections is not clear. Larger anastomoses are found at points where the main segmental curtaneous trunks cross the lateral nerve. The latter then has an uneven appearance, being thick in some places and at others very thin. The oldest specimen, which had lived thirty-one days after the operation, shows no continuous lateral nerve at all, though in places a few fibers are found. Thus in these cases, too, progressive degeneration takes place after excision of the ganglion cells, and it is not arrested even after the apparent establishment of anastomoses with other normal nerves of the vicinity.

The presence of connections between the degenerating r. lateralis and the intersecting general cutaneous nerves suggests the occurrence here of what has been observed by many investigators of nerve regeneration—the formation of connections between degenerating nerves and normal intact nerves in the vicinity which ordinarily do not anastomose.

Experiments made with the oldest stages used (larvae which had begun to feed) have unfortunately yielded nothing additional, because the process of degeneration is still slower and none of the specimens were kept alive long enough to show final results.

DISCUSSION

The experiments here described, together with observations on normal amphibian embryos, show that the development of the axone is a function of the neuroblast, and that the rôle of the Schwann cells cannot be formative. Motor nerves normally begin to develop without them, and certain sensory nerves, such as the Rohon-Beard dorsal nerves and some of the fibers from the spinal ganglia in the tail of the urodele larva, extend to the most outlying regions before sheath cells appear upon them. On the other hand, no such nerves are ever found that are not connected with central nerve cells. When the sheath cells are removed experimentally the sensory

nerves fail to develop, because the dorsal cells and the spinal ganglia are removed as well, but the motor nerves develop without sheath cells and likewise extend to their outermost normal limits. The removal of any body of neuroblasts, on the other hand, invariably results in the elimination of the peripheral nerves normally connected with them.

Until these results are shown to be erroneous, we must view with skepticism the newer descriptions of Götte ('14) and Pedaschenko ('14), as well as the older accounts in which the pluricellular origin of the axone is maintained. Such descriptions are based either upon insufficiently detailed observation or upon unsuitable and inconclusive material.

There are, of course, those who are skeptical of all embryological experiments and who regard the changes that take place after excision or transplantation of a part as abnormal and without definite relation to the normal processes of development. This objection has been admitted by experimental embryologists in so far as secondary regulatory processes have been recognized as occurring after injury to developing organisms.[33] The possibility of such processes having occurred may, however, usually be determined by comparison of the experimental results with the normal events of development. In the present case there is the closest agreement between the conclusions to be drawn from the two bodies of evidence. In particular, if one wished to invoke here an objection of the above kind, one would have to maintain, in the first place, that the neuroblasts in the medullary cord do not ordinarily form the motor axones, but that in the absence of the sheath cells, as in the ganglion-crest experiment, they may do so vicariously; secondly, that some sensory axones develop from chains of sheath cells, while others are formed only in connection with neuroblasts, such as the dorsal cells or spinal ganglion cells in urodeles; thirdly, that while certain peripheral motor nerves, e.g., those of the abdominal wall, are ordinarily formed from sheath cells, the latter are unable to form them when the ventral neuroblasts of the medullary cord are

[33] Cf. Roux ('94, p. 19).

removed. Thus we should be led to a series of inconsistencies, for which there is not a particle of evidence, whereas the assumption that the neuroblasts and not the sheath cells form the axones affords a simple and logical explanation of the facts. While the demand for a careful analysis of experimental evidence is altogether justifiable, the rejection of such evidence in toto amounts to nothing more than sheer obscurantism.

A further result of the present experiments is to show that the Schwann sheath cells are sui generis, and that they are not to be confused with mesenchyme cells, with which they may be closely associated. This is shown very clearly by the indifferent behavior of the mesenchyme cells toward the sheathless nerves, which often pass through long stretches of mesenchyme without exciting the latter to enter into any definite relation with them. On the other hand, sheath cells apply themselves closely to the nerve fibers, usually stretching out into spindle shape or otherwise adapting themselves to branchings of the nerves (figs. 30 and 36).

The question of the origin of the sheath cells is also answered by the ganglion-crest experiment. They are not mesodermal.[34] All of such cells present upon the spinal nerves up to the time when the frog embryo begins to feed are derived from the ganglion crest. In later stages apparently a few cells leave the medullary cord by the ventral spinal roots and probably (p. 161) become sheath cells. In the cranial nerves the placodes, as well as the ganglion crest, contribute sheath cells to the peripheral branches, and here the outward migrations of cells by way of the ventral roots seems to begin more precociously than in the spinal nerves. However, the experiments on the cranial nerves are not so conclusive as those dealing with the spinal. The difference between the amphibians on the one hand and the cyclostomes

[34] Formerly many investigators regarded these cells as originating from the mesenchyme, but opinion is now decidedly on the side of their ectodermal origin. The most recent writers who have expressed themselves in favor of the former view are Ramón y Cajal ('09) and Hoven ('10).

and elasmobranchs on the other, in respect to the migration of cells out along the motor roots, is thus one of the relative time at which the process begins.[35] On the whole, the sheath cells are best interpreted as a sort of peripheral neuroglia—an idea that has found favor with a number of students of the structure and development of the nervous system.[36]

While the recent work of Müller and Ingvar ('21) has fully confirmed the above results, Kuntz ('22), in a paper dealing mainly with the sympathetic system,[37] finds that after removal of the dorsal part of the neural tube and ganglion crest in a species of frog embryo, probably R. pipiens, the ventral roots, three and a half days after the operation, are accompanied by cells of medullary origin. Though it is of course possible, as Kuntz suggests, that there are specific differences in the time of beginning of this ventral migration of medullary cells, the evidence he gives does not prove his point conclusively. The cells shown in his photomicrographs might possibly be mesenchyme or vascular cells, or again, if sheath cells, they might have arisen from the dorsal half of the cord. Kuntz does not state how closely his sections were searched

[35] Held ('09, p. 142) states that he has never been able to determine that in the frog or axolotl embryo cells leave the medullary cord by the ventral roots. My own observations do not prove directly that they do so. One does, however, find in sections of specimens killed at the time when the yolk is entirely absorbed a few cells in the marginal veil close to the exit of the motor nerves just as we find them in the salmon embryo. Besides, older larvae from which the ganglion crest (exp. Z. 36 and Z. 56, p. 161) had been removed have some sheath cells in their motor peripheral nerves.

[36] Cf. v. Kupffer ('94, p. 75), Harrison ('04), Schiefferdecker ('06), Held ('09), Nageotte ('22, pp. 203, 307).

[37] The material at my disposal has not been favorable for the study of the sympathetic system, since the embryos from which the ganglion crest was removed have either been prepared by dissection or cut into slightly oblique longitudinal sections. Müller and Ingvar ('21) have, however, directed their experiments especially toward the study of this question and have found, that in the frog removal of the ganglion crest and the dorsal half of the spinal cord is followed by the total absence of the sympathetic, as well as of the spinal ganglia, while removal of the ventral half of the medullary cord does not prevent the development of either of these structures. In contradiction to this, Kuntz ('22, p. 13) finds that after the former operation "ventral nerve-roots accompanied by cells of medullary origin, as well as small primordia of the sympathetic trunks,

for the presence of sporadic ganglion cells and Rohon-Beard cells, either of which, if present, are a sure indication that all of the elements capable of giving rise to sheath cells were not excised. The single specimen of R. pipiens in my collection, subjected to this operation and later examined in sections, shows no essential difference from the conditions described in other species of frog; the ventral nerves are sheathless.

A further corroboration of the results here given is found in a very interesting observation by Dohrn ('07) upon an anomaly in a dogfish (Pristiurus) embryo. In this form the n. trochlearis receives its sheath cells normally from a branch of the trigeminal, the r. ophthalmicus minor. In the anomalous case in question this latter nerve was absent, and the n. trochlearis was found to be composed of naked axones, not a single sheath cell being present between the decussation of the nerve and the superior oblique muscle. This case is all the more important because it has to do with a different class of vertebrates from that dealt with in the present paper, and likewise with a different nerve.

are present in all the segments in which a considerable portion of the neural tube was left intact.'' Experiments on chick embryos made previously by Kuntz and Batson ('20) are also interpreted by these investigators as showing that the sympathetic ganglia are formed from cells that wander out directly from the medullary cord. After destruction of the ganglion crest and the dorsal part of the cord by electrolysis they found that sympathetic ganglia may develop in the absence of spinal ganglia. In rebuttal of this evidence, Müller and Ingvar ('23) have just published a second paper. These investigators have repeated the experiments of Kuntz and Batson on the chick and have obtained very clear cases in which well-developed motor roots were present, but both spinal and sympathetic ganglia lacking. Other cases, in which sympathetic ganglia are present while the corresponding spinal ganglia are either lacking or very minute, are explained on the ground that the ganglion crest was destroyed only after the first of its cells had migrated ventrally. The material of Müller and Ingvar was prepared by the Bielschowsky method, which stains nerve cells and fibers specifically and renders them distinct even in this difficult chick material. Their figures are detailed and clear. Kuntz has not used specific stains, and his schematic drawings and photomicrographs are not convincing. The weight of the experimental evidence is decidedly in favor of a common origin for spinal and sympathetic ganglia.

SUMMARY

1. Spinal motor axones first arise in Rana embryos with incipient tail-bud (fig. 1) as processes of neuroblasts situated in the ventral part of the medullary cord, each segmental nerve consisting at first of but one or two such processes. They are present in the anterior part of the trunk before the embryo shows muscular movement, and the individual segmental nerves develop successively in an antero-posterior direction.

2. While the motor nerves are developing, the ganglion crest breaks up and its cells migrate ventrally between the muscle plates and the medullary cord, and segregate in masses corresponding to the myotomes, thus marking the spinal ganglia at a very early stage. They pass as far ventrally as the upper part of the notochord, where they meet cells from the sclerotomes moving in the opposite direction. The newly formed motor roots (fig. 28) thus come into contact with these two cell groups almost at the beginning, though no definite relationship with them is established at first.

3. The motor nerves skirt the spinal ganglia on the medial side and as they extend farther ventrally between notochord and myotome, they are accompanied by spindle-shaped cells, which attach themselves closely to the axones.

4. The spinal ganglion cells delay for a considerable time in sending out their axone processes, the sensory spinal nerves being represented at first only by the nerves arising from the Rohon-Beard giant dorsal cells. The definitive dorsal roots and peripheral sensory fibers from the spinal ganglion cells do not appear until the embryo reaches a length of about 8 mm. and the external gills are formed (fig. 5).

5. As the ganglia and the corresponding myotomes shift posteriorly from their original position, the roots are considerably lengthened. At first the relation of the sheath cells to the motor roots seems variable; sometimes the root fibers are almost bare, again they are covered with sheath cells, as is the case with all ultimately.

6. The sheath cells are clearly separate elements, which are closely applied to the axones (figs. 30, 36, 39). While they have but a small amount of cytoplasm, they are in no sense mere nuclei and part of the axone itself.

7. As the sensory nerve fibers extend farther out into the fin folds, it is found in Rana embryos that the terminal stretches are devoid of sheath cells, although there are no axones entirely lacking such cells throughout their whole length. In the urodeles (Triton), on the other hand, there is a plexus of sensory nerves in the fin folds in young larvae about 9 or 10 mm. long, in which all of the nerves are sheathless. Some of these originate in the Rohon-Beard cells, others in the spinal ganglia.

8. Observations of the nerves in the fin fold of living anuran larvae show directly that the sheath cells migrate distally (fig. 9, A and B). The movement is evidently sporadic, cells remaining often for a long time stationary, and at other times (fig. 10) moving quite rapidly (35 μ in thirty minutes).

9. In nerve fibers which are degenerating after having been cut, the sheath cells after a day become irregular in shape (fig. 11) and somewhat granular, and may separate from the degenerating axone.

10. When the ganglion crest, together with the dorsal half of the spinal cord, is excised in embryos immediately after the closure of the medullary folds, the development of the spinal ganglia is prevented. Spinal motor nerves, which develop in such cases are naked axis cylinders entirely without sheath cells (figs. 32, 34, 35, 37, 38). Nerves of this kind may be followed, in appropriate preparations, to their ultimate distribution in the muscles most remote from the spinal cord (figs. 20, c, and 41).

11. In cases of incomplete removal, fragments of spinal ganglia develop in certain segments. The nerves of these segments have sheath cells in considerable numbers. There is thus a very close correlation between these two kinds of cells in their segmental distribution.

12. About the time the yolk is wholly resorbed sheath cells begin to appear even on nerves belonging to segments from which the ganglion crest was completely removed. This together with certain observations on normal specimens, indicates that in these later stages a few medullary cells migrate from the cord by way of the ventral roots.

13. After removal of the ganglion crest and the placodes of the head cranial motor nerves without sheath cells may develop (fig. 42), but in the case of these nerves medullary cells evidently begin to migrate along the ventral roots at a relatively early period, giving rise to some sheath cells, even though the other sources are removed.

14. Removal of the ventral half of the medullary cord, followed by the healing back of the dorsal half in place, results in the absence of nearly all motor nerves, the spinal ganglia and sensory nerves remaining intact. Purely motor rami of the segmental nerves, as, for instance, those innervating the primary abdominal muscle, are not formed in such cases, though the sensory nerves of the same region are normal (fig. 20, d).

15. Removal of the whole medullary cord of the trunk after the spinal ganglia are formed, leaving the latter partly intact, results in individuals with sensory nerves only. This, together with the foregoing experiment, shows that the sheath cells, which are present on the sensory nerves, do not form motor nerves vicariously in the absence of motor ganglion cells.

16. Cutting an embryonic nerve results, as in the adult, in the degeneration of the tract distal to the wound. In the case of the r. lateralis vagi, when the ganglion is removed at an early stage (6 mm.), the nerve undergoes no further development and all traces of it disappear in a few days. When the ganglion is removed at a later stage, development of the nerve likewise ceases, but degeneration takes place more slowly and the remaining sheath cells may form anastomoses with the nerves of the general cutaneous plexus which cross the degenerating lateral nerve.

17. The above observations and experiments show definitely and unequivocally that the sheath cells have nothing whatever to do with the formation of the axone and that the ganglion cell is the sole essential factor in its formation.

18. It is likewise definitely shown that the sheath cells are not mesodermal, but arise for the most part from the ganglion crest in connection with the spinal ganglia. Some arise later directly from the medullary cord by emigration along the ventral roots.

19. The sheath cells are to be regarded as the neuroglia of the peripheral nerves.

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PLATES

ABBREVIATIONS

ch, notochord
d.r, dorsal ramus
g.cr, ganglion crest
mes, mesoderm
m.c, medullary cord
m.r, motor root
mot.n, motor nerve
my, myotome
nbl, neuroblast
n.f, nerve fiber
n.oc.mot, oculomotor nerve
n.trigem, branch of trigeminus
oc, eye
p.a.m, primary abdominal muscle
s.c, sheath cell
sen.n, sensory nerve
s.r, sensory root
sp.g, spinal ganglion
v, blood vessel
v.r, ventral ramus
y, yolk

PLATE 1

EXPLANATION OF FIGURES

27 Portion of cross-section through the trunk of an embryo of R. sylvatica in stage at which the ganglion crest was removed. Series 4a, row 9, last section. × 158.

28 Portion of frontal section, in the region of the fourth myotome, of an embryo of R. sylvatica at a stage just before muscle contractions begin. Large motor neuroblasts are shown, one of which continues into a nerve fiber ($n.f_1$) running to the myotome (*my4*). Series 5b, row 6, section 7. × 420.

29 Portion of a sagittal section through an embryo of R. palustris, 11 mm. long. A large sporadic bipolar ganglion cell on the ventral ramus of one of the nerves near the base of the tail is shown, also relations of sheath cells (*s.c.*) to the nerve fibers (*n.f*). Experiment Y. 25 (control). Slide 2, row 2, section 8. × 420.

30 From a sagittal section through an embryo of R. palustris, 9.5 mm. long (12c). A spinal ganglion (*sp.g*) and nerves from between the myotomes near the base of the tail are shown. Sheath cells (*s.c*) are present as distinct cells lying upon the fibers (*n.f*). Series 12c, row 8, section 5. × 420.

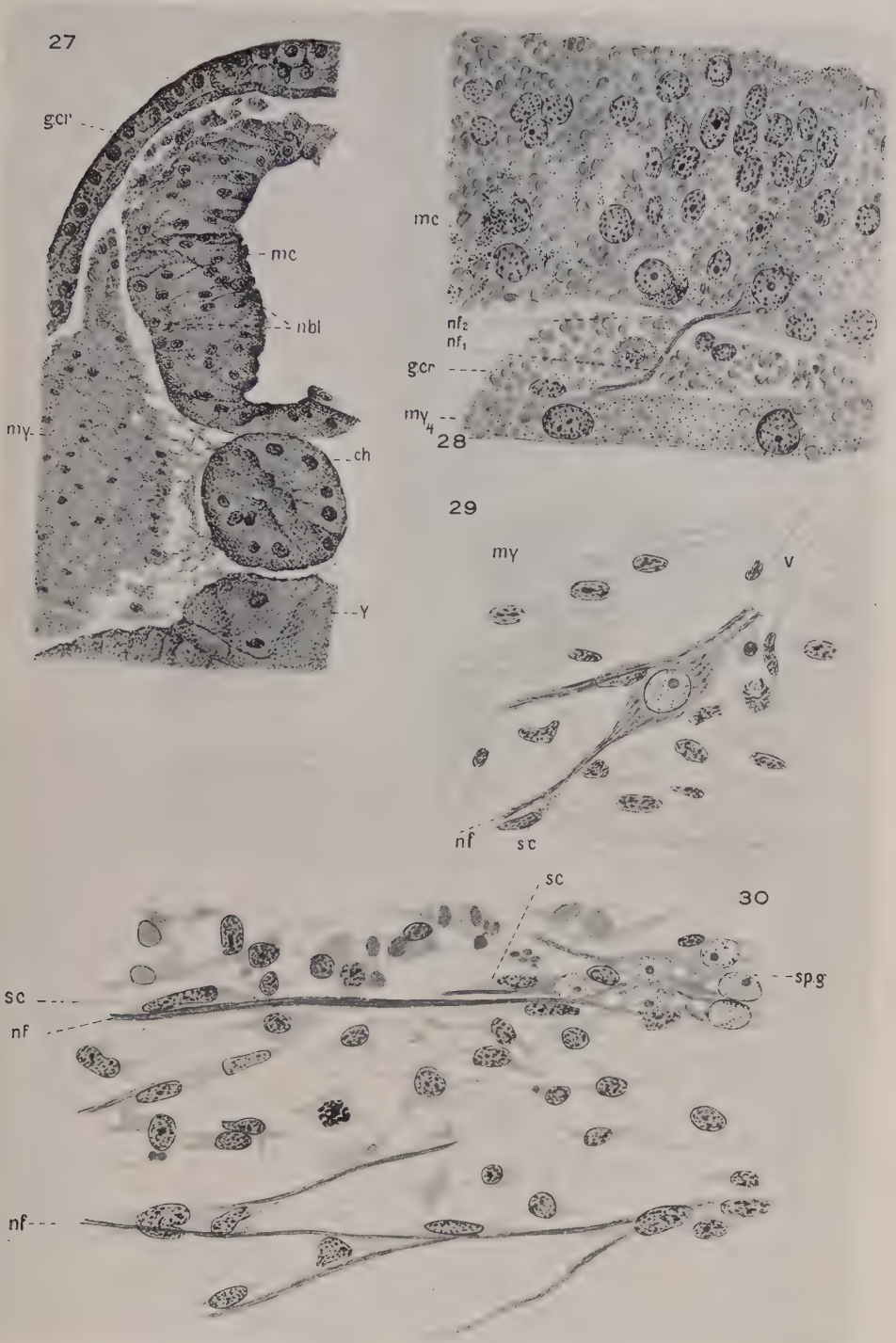

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PLATE 2

EXPLANATION OF FIGURES

31 Typical spinal nerve showing motor (*m.r*) and sensory roots (*s.r*), spinal ganglia (*sp.g*) and dorsal (*d.r*) and ventral rami (*v.r*). The nerves have numerous sheath cells. Combined from three sections of an oblique sagittal series of a Rana sylvatica embryo, 11.5 mm. long. Experiment Z. 77 (control). Row 4, sections 7, 8, 9. × 158.

32 Spinal nerves in a double larva from which the ganglion crest had been removed. The two components were grafted together back to back. The motor roots to each are seen leaving the combined medullary cord. The nerves are devoid of sheath cells. From a section cut slightly obliquely to the sagittal plane. Experiment Z. 77 (R. sylvatica). Row 10, section 5. Specimen preserved nine days after operation. × 158.

33 Twelfth spinal nerve of one of the components of experiment Z. 77. A small spinal ganglion (*sp.g*) has developed owing to incomplete removal of the ganglion crest. Sheath cells were present on the ramifications of this nerve. Row 10, section 3. × 158.

J. F. CHANDLER ET R. G. HARRISON DEL.

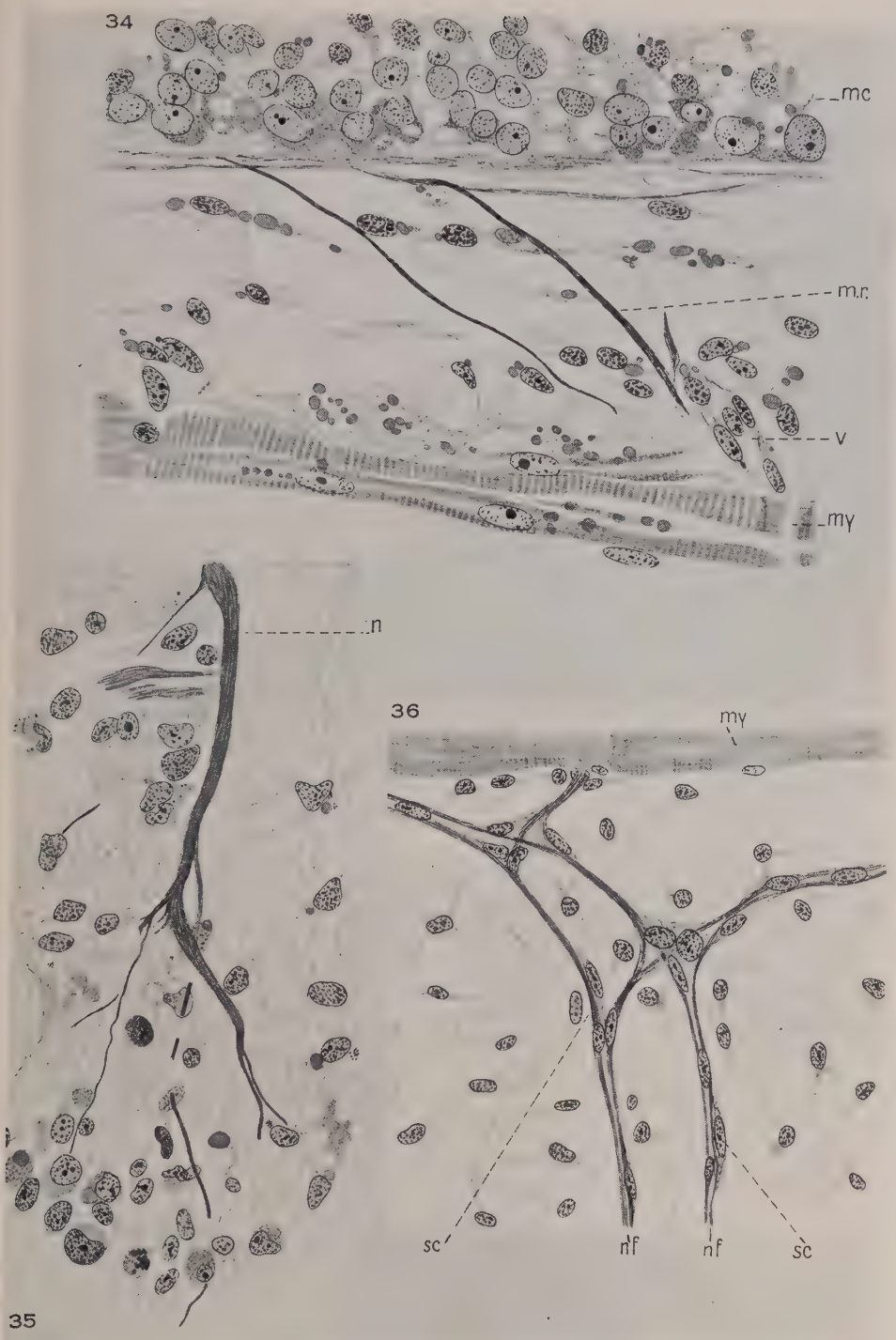
PLATE 3

EXPLANATION OF FIGURES

34 Portion of a slightly oblique sagittal section of an embryo without ganglion crest, showing the origin of the motor roots of a spinal nerve. The nerve fibers are sheathless. Experiment Z. 19 (R. esculenta); specimen preserved five days after operation. Row 13, section 5. × 420.

35 Ventral ramus of spinal nerve (*n*) of the lumbar region of a larva from which ganglion crest had been removed; no sheath cells present. From an oblique sagittal section of a specimen preserved seven days after operation. Experiment Z. 65 (R. palustris). Row 16, section 12. × 420.

36 Similar nerve from a normal embryo showing numerous sheath cells. Experiment Z. 77, control (R. sylvatica). Row 3, section 1. × 420.



R. G. HARRISON DEL.

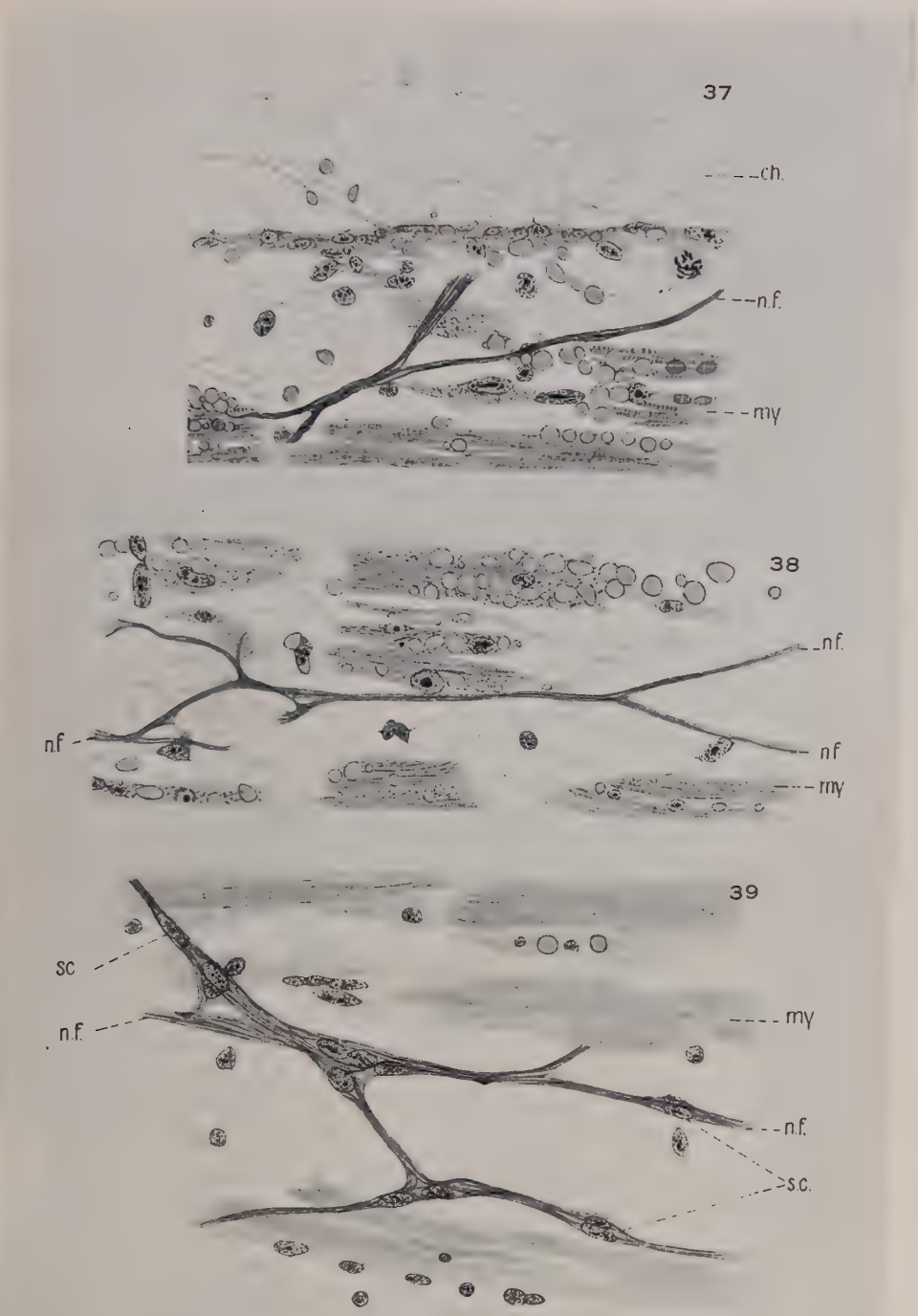
PLATE 4

EXPLANATION OF FIGURES

37 Portion of sagittal section of larva without ganglion crest, showing motor nerve plexus near base of tail; no sheath cells present. Specimen preserved nine days after operation. Experiment Z. 77 (R. sylvatica). Row 9, section 2. × 420.

38 Another portion of plexus from same specimen as figure 37. Experiment Z. 77. Row 10, section 4. × 420.

39 Similar preparation from the normal control larva, showing numerous sheath cells (*s.c*). Experiment Z. 77 (R. sylvatica). Row 3, section 8. × 420.



J.F. CHANDLER DEL.

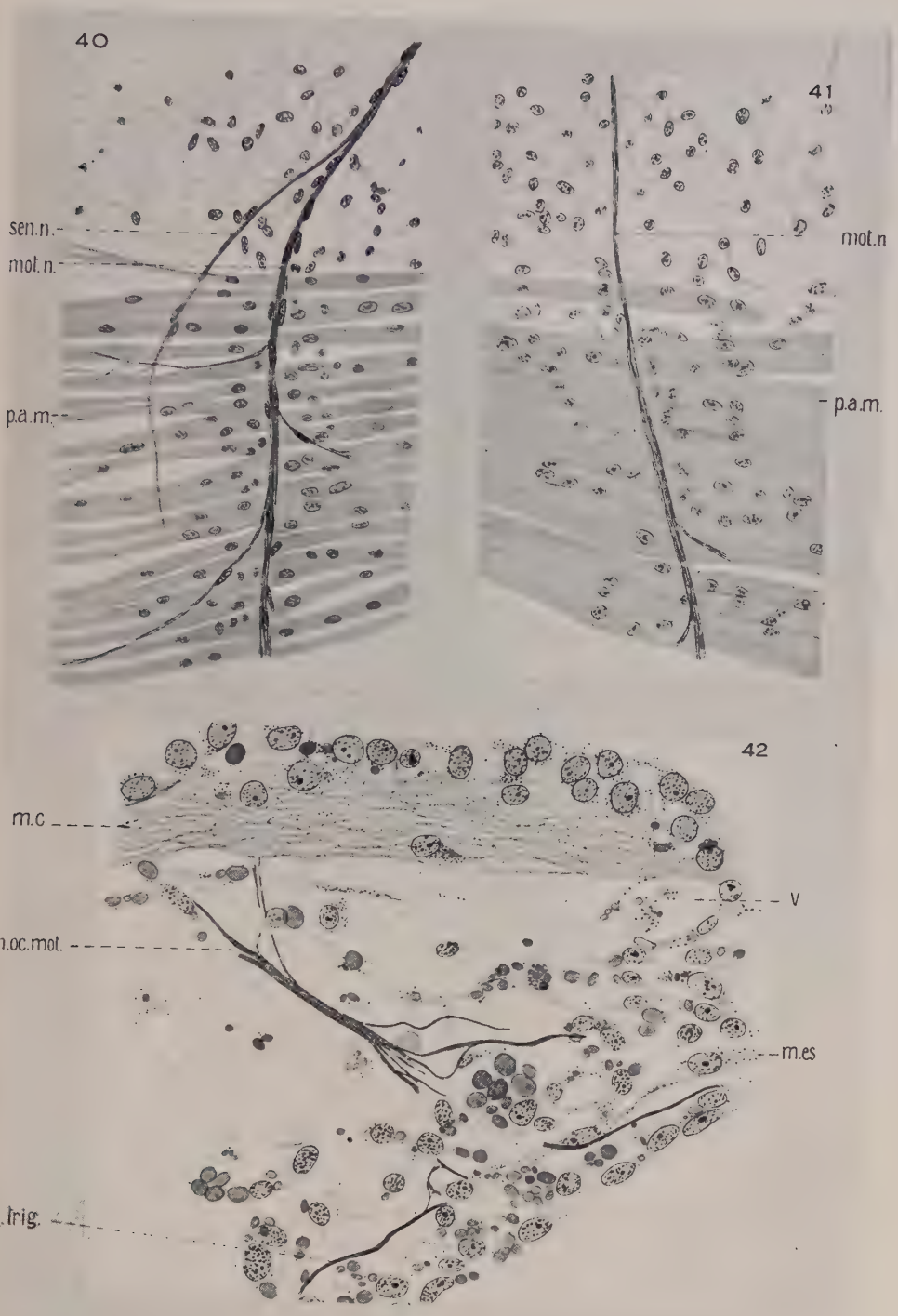
PLATE 5

EXPLANATION OF FIGURES

40 From an abdominal wall preparation of a normal larva of R. palustris about 10 mm. long. The abdominal ramus of a spinal nerve divides into a motor branch (*mot.n*) running internally to the muscle along an inscriptio tendinea, and a sensory branch (*sen.n*) running externally to the muscle. Experiment Z. 94, control. × 210.

41 Similar preparation from a larva without ganglion crest. Only the motor branch is present, and this is without sheath cells. Experiment Z. 94 (R. palustris). Specimen preserved eleven days after operation. × 210.

42 Portion of a sagittal section through the head of an embryo from which ganglion crest and placodes had been removed. What is probably the oculo-motor nerve (*n.oc.mot*) and also an anastomosis with the trigeminus (*n.trigem*) are shown, both without sheath cells. Experiment G.S.O. 5 (R. palustris). Specimen preserved four days after operation. Row 4, section 3. × 420.


40
sen.n.
mot.n.
p.a.m.
41
mot.n
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42
m.c
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R.G.HARRISON DEL.

A STUDY OF THE OLFACTORY SENSE OF TERMITES.

(Twenty-four Figures).

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Introduction.

The main object of this study has been to investigate the olfactory sense of the termite *Reticulitermes flavipes* (Kollar), with the purpose of subjecting to analysis McIndoo's assertion that the olfactory sense in insects is not located on the antennæ but on the legs and wings. While McIndoo and others have published a great deal on the olfactory sense of various insects, nothing has been done on the "white ants." The only work on termites which is at all related to this subject, is that by Stokes, dealing only with the morphological aspect and that only superficially. His observations regarding the distribution and external structure of the pores upon the legs agree in general with those in this paper though here a greater variation was found in the number of the pores. His paper is concerned in large measure with the morphology of the hairs and bristles, while this paper touches chiefly upon the morphology of the pores and short hairs (pegs) which might possibly be concerned with the sense of smell. He makes one extremely interesting observation which seems worthy of attention even though he does not lay much stress upon it. To quote, "with these organs, the termite is right well prepared for whatever may happen, even for the forceps of the predatory microscopist.

The forceps conquers in the end, but the insects seem to feel its presence before it touches them, retreating and sometimes backing away from it as from some obnoxious object."

It does not seem necessary to give a lengthy account of McIndoo's work, since this is quite generally known. He has studied in great detail the olfactory sense in Hymenoptera, Coleoptera, Lepidoptera, and Diptera, both from the morphological and experimental standpoints. Hicks, in 1857, described peculiar pore-like organs on the wings and halters of flies and on the wings of many other insects. In 1860 he discovered the same "vesicles" on the trochanters and femurs of all the insects he examined. He made the suggestion that these pores, because of their delicate structure, might, in some way, be connected with the sense of smell, or perhaps with some sense not found among Vertebrates, but he did not perform any experiments to substantiate his views.

McIndoo's work has been a detailed continuation, among other groups of insects, of the morphological study begun by Hicks, and in addition he has performed countless experiments by which he believes he has proved beyond question olfactory function of the pores.

Recently, however, many objections have been raised by Karl von Frisch in a paper entitled "Uber den Sitz des Geruchsinnes bei Insecten," in which he describes a series of very careful experiments upon bees, the results of which seem to indicate that the olfactory sense is located, not upon the legs and wings, but upon the antennæ. His new experimental methods will be discussed later, in connection with those of McIndoo, and the ones with which this paper is chiefly concerned, on the termites.

The study of this problem was carried on under the direction of Prof. Alexander Petrunkevitch, to whom I am greatly indebted for much of the material, as well as for many helpful suggestions and criticisms.

Materials and Methods.

For the study of the disposition and the morphology of the pores, specimens were at first boiled in potassium hydroxide, but it was soon found that specimens preserved in 70% alcohol and mounted in glycerine were much more satisfactory.

For the internal structure of the pores, live animals were fixed in Freihling's solution, heated to 40 or 50 degrees Centigrade. Other animals were fixed in Vom Rath's fluid, which is excellent for showing the nerves and the nerve cells. All specimens were cleared in cedar oil and imbedded in 45 or 54 degree paraffin. The posterior parts of the bodies of the termites were usually cut off in order to facilitate infiltration. For the study of the pores on the trochanters, single legs were oriented and imbedded separately, and sectioned at 5 μ. Sections in most cases were cut from 4 to 8 μ. in thickness. Sections fixed in Vom Rath's fluid seldom required staining, though in one or two cases where necessary, they were satisfactorily stained with Delafield's Hematoxylin. Sections fixed in Freihling's solution were stained in several ways. Iron Hematoxylin counterstained with Orange G being used most frequently.

Morphology.

Disposition.

In making a survey of the Hicks pores, about twelve workers were used. Specimens of soldiers and winged forms were examined for comparative purposes.

For convenience in discussion, the groups of pores have been numbered. Groups 1 and 2 are found on the trochanters of all the legs, Group 1 on the ventral side, Group 2 on the dorsal side. Group 3 is at the proximal end of the dorsal surface of the femur. Group 4 includes all the pores found on the antennæ with the exception of certain pores labelled 4a, which will be discussed later. Groups 5 and 6 are located on the mandibles, Group 5 on the dorsal side and Group 6 on the ventral. Group 7 is comprised of three pores found on the ventral side of the second segment of the maxillary palps. Group 7a are scattered pores and pegs on the distal edge of the galea of the maxilla. Group 8 refers to a single row of pores found along the galea of the labium on the dorsal side, and Group 9 to those more irregularly placed pores of a similar nature on the ventral side. The two marked rows of pores on the ventral surface of the labrum are called Group 10. There is a small group of pores on each side of the ventral surface of the hypopharynx, Group 11.

Very definite groups of short peg-like hairs have been observed, such as Group a, on the ventral median surface of

the coxæ, and Group b, on the trochanters at their articulations with the coxæ, on the concave side. Similar groups are found on the maxillary and labial palps at the basal portion of each segment close to the articulation. Those on the maxillary palps are called c_1, c_2, c_3, c_4, and c_5. Those on the labial palps are d_1, d_2, and d_3. Other groups of these hairs are present at the bases of the maxillæ, called c_6 and c_7. The small group at the very bases of the antennæ is referred to as Group f. There are some hairs covering the labrum which appear different from these other groups of peg-like hairs in that they lie extremely flat. These are called Group e.

These pores vary between the different groups in size and appearance, but the pores of the same group on different animals vary only slightly and then mainly in size, though there is some slight variation in the numbers in some of the groups. There is a definite gradation in the sensory structures, beginning with small, round pores without any conical elevations, to long, extremely stiff spines, such as are found on the edges of the galea of the maxillæ. At certain places on the body are found large, perfectly round pores which give the appearance of being the bases of medium sized hairs (e) after the hairs have been lost. Such, for instance, are the pores of Group 4a. Text figure 1, a and b, are diagrams of the appearance of the pores, and are found in Groups 1 through 11. Type b is the typical Hicks pore, such as is found in definite groups on the trochanters of the legs.

Pores on the Legs.

(Plate XIX, Figs. 1–8).

The distribution and type of the pores are the same on all the legs. Group 1 is found on the ventral side of the trochanter of each leg. This is the largest group and is a constant one. It is subdivided, in that four of its pores are a little to one side, lying in a row by themselves next to the line of articulation with the femur. Four is the usual number of this subgroup, but it is found to vary to a small degree, generally by not more than one. The other part of the group is distributed irregularly over the rest of the ventral side. This subgroup consists usually of ten pores, but may occasionally have as many as thirteen. All the pores of Group 1 are almond shaped, that is, somewhat elongated and pointed at each end. They are easily found with the medium and high powers of the microscope.

Group 2 is found in the position corresponding to that of Group 1, but on the dorsal side of the trochanter. This is a much smaller group of the same type of pores. The number of these is usually five, though in some cases four or six were present. These are as close together as those in Group 1, but being fewer in number, cover only a small portion of the dorsal surface.

Group 3 is very small but constant. It is at the proximal end of the femur close to the articulation of the femur with the trochanter, on the anterior dorsal surface. These vary in number from two to five, but they correspond on opposite legs. If the front left leg has three of these pores, the right front leg will possess the same number. The front pair of legs, however, may have three pores in this group, while the middle pair may have five, and the hind pair four.

Other pores have been seen on other parts of the legs but these are never constant and so are mentioned merely as being occasionally present. Such scattered pores are seldom of the type described above, but are the large round flat type which have been noticed on all parts of the body and which look as though they might be the bases of lost hairs. If these were constant, they might possibly be functional pores.

Pegs on the Legs.

There are certain groups of peg-like structures on each leg which should be described even though they may not have anything to do with the olfactory sense. These have been referred to as Groups a and b.

Group a is constant and is located on the ventral side of the coxa of each leg, close to the place where it articulates with the body. This is a group which at first sight might be taken for pores, because they appear as small bright spots in strong light. On focusing up, however, they were seen to possess extremely short processes like pegs. These short processes are about twice as long as their bases are wide, (type d). There are, on an average, ten of these structures, though they vary from nine to twelve.

Group b consists of similar structures on the ventral surface of the trochanter of each leg close to the articulation between it and the coxa. These vary in number from seven to twelve, with an average of ten. They are identical in structure with

those of Group a, and are very closely packed together. They are sometimes difficult to find because they are right in the bend of the leg, more anterior than ventral.

Pores on the Antennæ.
(Figs. 9 and 10).

The antennæ of the termite worker consist of sixteen segments. All of the pores on them have been called Group 4, though they are by no means arranged in a group. They are, however, constant in their location. On the dorsal side of each antenna, near the base of each segment, is found one single pore similar to those on the trochanters of the legs except that they are somewhat smaller, and, though a little elongated, are rounder. (Text Figure 1, a). A corresponding pore is found in the same position on the ventral side of each segment. On the dorsal side of the basal segment near the articulation of this segment with the second one, are two pores side by side. In one animal, three such pores were seen at the anterior part of the second segment, on the dorsal side. At the basal part of the first segment of the antenna on the distal side, close to the place where the antenna joins the head, is a small group of peg-like hairs such as were described on the legs (Group f). The pegs of this group vary in number from three to six. A similar group is sometimes present at the base of the second antennal segment.

Two long hairs are present both on the dorsal and on the ventral sides of the antennæ near the middle of each segment. In between these with many more to the anterior end of each segment, are smaller hairs which are about half the length of the longer ones. On some of the antennæ there appeared to be large perfectly round pores at the places where the two long hairs ordinarily were found. In some of the first specimens, there were so many of these that it seemed as though they were constant and were perhaps sensory pores, but the examination of more termites has led to the conclusion that these cannot be more than the bases of lost hairs. The hairs were found to be attached on some and absent on others. They were seldom absent from the same places on different segments. Some specimens were seen where all the hairs were present on all the antennæ and none of these round pores were observed. The small round true pores are, however, constant, though so few in number.

Mouth Parts.

Labrum.

(Plate XX, Fig. 15).

The most conspicuous pores on the labrum are found on the ventral side which is next to the other mouth-parts, fitting closely over the mandibles. These compose Group 10, and are arranged in two rows extending the entire length of the labrum, one on each side of the center line. They meet at the very anterior edge, forming a horse-shoe. The rest of the inner ventral surface is covered with very short fine hairs which lie extremely flat and all of which point toward the anterior midline. At the anterior edge of the labrum is a narrow row of bristles which project a little beyond the margin. Longer hairs are present in a fan-shaped arrangement at the articulation of the labrum with the palate. The pores of Group 10 appear flat when focused upon it one way, but when the lens is focused up they seem to be slightly raised, (Text Fig. 1, c). There is present quite a definite gradation of hairs into pores from the outside edge inwards. From the row of pores toward the midline, they grade back to short hairs.

Mandibles.

(Plate XX, Figs. 11–14).

Large groups of pores, Group 5 on the dorsal side, Group 6 on the ventral side, are found on the mandibles. These were examined in about eight specimens and were found to be extremely variable in both the number and position. They are not in any definite groups but are scattered about over the surface of the mandibles, usually nearer the articulation of the mandibles than the heavy chitinous rim. On the ventral side they are somewhat more separated into what might possibly be termed groups, than on the dorsal side. Here, in four specimens, were found variable groups of larger pores, identical in appearance with the pores on the legs. Other smaller pores of the type a were scattered over the rest of the surface. On the dorsal side of the mandibles are found a large number of these same small pores, but in with these are distributed a few of type d, very short peg-like hairs such as have already been described on the legs. The number of the different types is

variable. Group 5 on the dorsal surface of the right mandibles varies from 10 to 16, with an average of 14. The pegs vary from 2 to 8 with an average of 5. Group 5 on the dorsal surface of the left mandibles varies from 5 to 10 pores, with an average of 7. The pegs vary from 5 to 9 with an average of 6. At first it seems strange that there should be such a wide variation between corresponding parts, but it does not seem quite so strange when it is seen that in no case is one mandible the mirror image of the opposite mandible of the same animal. In termites of different species the mandibles of the same animal are often quite different from each other.

Group 6 on the ventral side of the right mandibles varies from 10 to 15 with 13 as an average. On one of the mandibles there were 15 present but 10 were apparently pores of type b, and 5 were of the smaller variety. On the ventral side of the mandibles these two types were seen more often. The large pores varied from 5 to 9, 7 on the average, and of the small pores there were usually 8 or 9. No peg-like hairs were ever seen on the ventral sides.

When the microscope was focused down on these pores, their passage through the thick chitin to the very interior could be clearly observed.

Maxillæ and Maxillary Palps.

(Plate XX, Figs. 18 and 19).

The only true Hicks pores on the maxillæ are found in groups of three (Group 7) on the palps at the ventral distal portion of the second segment. Occasionally Hicks pores may be found isolated on other parts of the maxillæ but these are never constant.

Group 7a on the ventral side of the galea, consists of 9 to 12 very minute pores or pegs. The elevations of these are so small that they are seen only with the most careful observation. On some, the tiny raised hairs could not be seen at all, and it may be that this group is a mixture of the pores of type a and the small pegs of type d. On the dorsal side of the galea are found small pegs. These are a little longer than the ones on the ventral side and there are not as many of them.

At the base of each maxilla on the ventral side, there are found two groups of pegs (Groups c_6 and c_7). Group c_6 is more proximal than Group c_7 and they are also very slightly longer.

Pegs are found in definite small groups c_1, c_2, c_3, c_4, and c_5, at the articulations of the segments of the palpi. These are seen from the dorsal side. They are short, very slender pointed hairs of type d, exactly the same as those described on the legs and at the bases of the antennæ.

On each segment of the palpi there are small round pores of the type a, such as are found on the antennæ, but they are not definitely located.

Labium and Labial Palps.

(Plate XX, Figs. 20 and 21).

Group 8 consists of a definite row of the pores of type a, which are situated on the dorsal side of the galea of the labium, this being really the floor of the mouth. This row of pores extends from the very distal tip end to the base where the labium articulates with the head proper. Group 9 is composed of similar pores on the ventral side, but these are not in any such definite arrangement, being scattered over the length of the galea. On the ventral side of the lacinia are a few scattered small pores and pegs.

Groups d_1, d_2, and d_3, consist of small pegs of the type d such as were present in similar groups on the legs, the maxillary palps, and at the bases of the antennæ. On the labial palps they are found at the bases of each segment near the regions of articulation, just as on the maxillary palps. Each group is composed of six or seven pegs.

Hypopharynx.

(Plate XX, Figs. 16 and 17).

The dorsal surface of the hypopharynx is covered with hairs which lie very flat and are arranged in two longitudinal rows with the hairs directed toward the midline. At the basal part they are in a rosette formation. The ventral surface is comprised of similar hairs arranged as indicated in Figure 17. On either side of the midline near the articulation are four true Hicks pores, Group 11. None of the pores of type c such as were seen on the labrum, were observed on the hypopharynx.

External Structure.

Pores of type b, such as are most numerous on the legs of the termites, are the true Hicks vesicles. They have a definite

shape and would never be confused with the pores of type a on the antennæ, or with the bases of hairs. They are almond shaped, elongated and pointed at opposite ends. The greater part of the external surface is flat but there is a narrow darker boundary encircling the edge. This appearance may be due to the internal structure of the flask. The manner in which the sides of the flask curve makes a greater thickness of chitin at the edges through which the light must pass when examined under the microscope. On the legs of the soldiers the pores seem to have slight elevations. It may be that the "chitinous cones" do not fit closely against the base of the flask. The pores on the antennæ are elongated to a small degree but would never be confused with the true Hicks pores because of their smaller size. Most of the pores on the mandibles, maxillæ, labium, etc., are quite round and very small, though on some mandibles, on the ventral side were seen pores more nearly resembling the true Hicks vesicles. External apertures were not observed on any of the pores though it cannot be said that they are never present. It is possible that the nerve fills the aperture so that, externally, any such opening could not be noticed.

Internal Structure.

(Plate XX, Figs. 22–24).

As in many other insects whose pores have been studied, the Hicks vesicles are small "inverted flask-like structures," the base of the flask being the chitinous layer. If a pore aperture is present, it would be in the center of this layer. At the bottom of the flask, as in the pores of most insects, is a closely fitting plug of chitin which McIndoo calls a "chitinous cone." In the sections fixed in Vom Rath's fluid, a sensory fiber could be traced to this "chitinous cone," but in no sections could it be seen to perforate it. This is not to be wondered at, however, for the pores are extremely minute. In order to determine exactly all points regarding the internal structure of these pores, a great many slides would have to be made before one could be found which would pass through the pore aperture if such were present. In the works of several German investigators to which Frisch refers, no apertures were represented and Frisch is inclined to disregard the statements of McIndoo on this point. How odors can pass through the chitin without some such arrangement is difficult to comprehend. Experiment is surely

a much sounder basis for determining the location of any sense than a morphological study ever could be.

Structures on Soldiers and Winged Forms.

There is really very little to be said regarding the location and the numbers of the various groups of pores and pegs on these two castes because, in general, they possess the same groups with only the slightest variation in the numbers within the groups. Consequently, only the outstanding differences will be mentioned.

There is one noticeable difference in the external appearance of the pores on the trochanters of the soldiers from those on the workers. They have a definite elevation which was found to be due to the presence of a sort of raised hood-like membrane. It was quite impossible to tell even under the highest power, whether each of these pores had an external opening or not. The spot in the center of each one might easily have been the ending of the nerve inside, or the hollow internal area of the flask.

Stokes, in his paper, states that the pores of the subgroups of Group 1 are of different structures, the ones in the center being "conical, often oblique, hood-like structures" while the group of four at the side resemble "flat-topped papillæ pierced with a central depression above the membrane." In all the termites examined for this study, no such difference has been noted. All the pores on the trochanters of the legs are the same, except that in the soldiers the membrane over the top of each pore is elevated to a greater extent than in the workers.

Since the mandibles of the soldiers are quite different from those of the workers or the winged forms, a special examination was made. There was found to be just as great a variation in the number of the pores and pegs, and in their disposition, as on the mandibles of the workers. They are scattered about over the dorsal and ventral surfaces in quite a haphazard way. There may be three to five groups. It is really hardly safe to call them groups. However, the same thing is true here as in the case of the worker, namely, that while pores and pegs are interspersed with each other on the dorsal side, there are never any pegs on the ventral side.

On the maxillæ of the soldiers, on the ventral side at the basal part, are many minute pegs and in among them, in one case, were seen two true Hicks pores.

The wings of the winged caste were examined for the purpose of discovering whether or not groups of pores were present near their bases as have been described by Hicks and McIndoo in so many other insects. At most, there were only a few scattered pores, the bases of the wings being covered with many hairs. There was no indication of any such definitely arranged pore groups as those seen, for instance, by McIndoo on the ants, bees, and flies.

Experiments.

Review of Previous Work.

McIndoo has studied in detail, the structure of the pores which were first described by Hicks. He finds organs on the antennæ of bees which externally appear much the same as the pores on the legs and wings. These he calls "pore plates." A study of the internal structure of the "pore plates" shows that if they are olfactory in function, the odors must pass either a thin or a thick layer of chitin in order to reach and stimulate sensory nerve fiber. Some authors have attributed an olfactory function to these organs in spite of their structure, by assuming that odors can pass through the hard chitin in insects where it would be impossible in higher animals.

The "olfactory pores" on the legs and wings, he finds much more adapted for service as olfactory organs because, as he says, the nerve fibers run to the external surface where they can be stimulated by odors without it being necessary for the odors to pass through the hard chitin. This is one of McIndoo's arguments for his conclusion that the olfactory sense in insects is located on the legs and wings and not on the antennæ.

Frisch disagrees with McIndoo as to the structure of these pores. He says that anyone who is acquainted with their morphological structure as described by Leydig (1860), Kraepelin (1883), Weinland (1891), Rohler (1906), Vogel (1911), Hochreuther (1912), or Deegener (1912), could believe that the pores on the legs and wings are better adapted for an olfactory function than those on the antennæ. He does not agree with McIndoo's assertion that in the "olfactory pores" (vesicles of Hicks), the chitin is perforated by the nerve ending and so comes into direct contact with the air. Frisch emphasizes the fact that the only way to be at all sure what organs are olfactory, is

by new experimental methods, since we know nothing concerning the permeability or impermeability of the chitinous membranes to olfactory particles.

While McIndoo lays much stress upon the morphological structure of the pores, he has tried to uphold his views with a series of detailed experiments. Heretofore, most of the investigators in this field, had paid no attention to the normal behavior of their insects. They cut off a few joints of each antenna, the whole of both antennæ, or varnished them with shellac, paraffin, vaseline, and other substances. Then when the reactions of the insects were weakened or discontinued entirely, it was unhesitatingly concluded that the organs for the perceptions of odors were located on the antennæ. It is quite natural that they should reach such a conclusion because most of the insects thus mutilated have failed to respond to odors held near them to which they would ordinarily have reacted. They have failed to find food, and when removed from food they have accidentally come across, have failed to return to it. Males so mutilated do not usually seek females and take no notice of females placed near them.

McIndoo criticizes all these former experiments, saying that the men have left out of consideration the normal behavior, and that they have disregarded the effect upon the insects, of the mutilations. Miss Field (1901) is the only one who has kept any record of the effect of the mutilations upon the normal lives of her insects. According to her, only 40% of her mutilated ants recovered from the shock, and, in all probability, she says, none of these were absolutely normal. McIndoo has kept records of the effects of such operations upon the behavior and length of life of his animals. He claims that cutting the antennæ injures the insect to such an extent as to make it impossible for the animal to respond to odor stimuli. Exposing the large sense cells to the air would, in itself, cause abnormality. Furthermore, his records seem to show that such mutilations shorten the lives of the insects. He admits, however, that as long as they do live, they appear to be perfectly normal, except in their failure to recognize odors.

McIndoo's first experiments were performed upon normal bees. As odor stimuli he used the essential oils of peppermint, thyme, and wintergreen, bee foods, such as honey, honey comb, and pollen, parts of plants such as honeysuckle flowers, penny-

royal, spearmint, and scarlet sage, and also bee stings. His conclusion from these experiments is merely that bees possess an acute sense of smell.

Having become thoroughly acquainted with the reactions of normal bees, McIndoo proceeded to experiment upon mutilated ones. When one or both antennæ are removed, they apparently recover from the shock and seem normal, but they do not live as long as the unmutilated ones. But since they are shorter-lived they must be abnormal and consequently he believes that any results obtained from experiments upon them cannot be regarded as valuable. Bees having both antennæ pulled off or covered with celloidin, failed to respond to the essential oils. When both the antennæ were burned off, seven of the bees thus treated, recovered sufficiently to eat, though they were far from being normal. They readily responded to the essential oils, the reaction time being not quite twice that of normal bees.

From these experiments McIndoo draws his conclusions. Whenever the antennæ are mutilated in any way, the insects are never normal. Since they are abnormal, if they fail to respond to odors it can be laid to the effect of the operation. Moreover, since seven bees recovered to a slight degree and did respond, the olfactory organs must be located elsewhere than on the antennæ.

Animals having their maxillæ or labial palps removed reacted normally, though they did not live as long as unmutilated ones. The same was true when the proboscides or mandibles were cut away. McIndoo, therefore, does not believe that the olfactory organs are located on the mouth parts. Considering that the pores described by Hicks on the legs and wings are conveniently situated and are so evidently adapted morphologically for the reception of some kind of chemical stimulus, he decided to test them for what seemed the most probable, that of odor.

When the wings of the middle aged bees were removed, the reaction time was increased eight times. When the pores on the legs were covered, the time of response was increased twelve times. Since these animals were entirely normal in their behavior and length of life, this increase in reaction time could not be laid to abnormality and therefore McIndoo's conclusion is that these pore-like organs are to some degree

concerned with the sense of smell. By similar experiments upon beetles and ants he reaches the same conclusions.

Exactly opposite conclusions as to the location of the olfactory sense in insects, at least in the bee, have been reached as the result of the more recent experimental work carried on by Karl V. Frisch. Frisch is extremely skeptical regarding the conclusions held forth by McIndoo. As stated above, he does not believe that the pores on the legs or wings are any better adapted for receiving odor stimuli than the "pore plates" on the antennæ. McIndoo considers them superior because the nerve endings perforate the chitin and are exposed to the air. Frisch does not admit the presence of any such apertures. He does not think it wise to base conclusions as to the location of any sense upon ground less firm than that of experiment. He therefore looks to new experimental methods to solve the problem. The new method adopted by him for this purpose is one in which all the bees used in the olfactory experiments are first trained to recognize certain odors. Instead of using the irritating essential oils, he employed such odors as esteric oils, flowers, etc. He saturated the food of the bees with some odor and then trained the bees to come to it regularly. He used several square glass jars or boxes and in one only the fragrant food was placed. The bees came to this box invariably for their food. To this one jar was fastened a feeding trough which became automatically filled when the bees entered it. During the experiment the feeding trough was taken from the scent box, placed on top, and the box removed to a new location. The bees flew about for some time until one bee noticed the change. As soon as it drank it was marked on the thorax and placed with the trough into the box. The bee soon departed, but learned to return to the new place, bringing other bees which were also marked. During this experiment, the box containing the odor was often changed about from one place to another and boxes identical in appearance but containing no odorous substance within, were substituted. After the bees were sufficiently trained so that they never failed to find the odor, their antennæ were cut off. Immediately they were unable to locate the scent box. Of eighteen experiments none failed. Covering the antennæ with oil, paraffin, or shellac gave poor results.

Frisch carefully controlled all his experiments with color experiments. He used, for this purpose, boxes of exactly the same size and shape, but of different colors. The bees were trained to come to one box. When the antennæ were cut, they were in no way hindered from finding the colored box. In this case the bee is controlled by sight, while in the former experiments by smell.

When a single antenna was removed, there was found to be practically no effect upon the ability of the bees to find their food. When a few segments of each antenna were removed the bees showed plainly that they recognized odors. If eight distal segments of the twelve which make up antennæ were cut off, the bees acted as though they had no antennæ at all. No olfactory organs are then located upon the three basal segments. If the antennæ were cut so that on one side four segments remain, and on the other side only three, even then the bees were able to recognize the odor. Moreover, a bee in which only five segments of one side remained, could still be trained to recognize a new odor. The only conclusions to be drawn from these experiments is that made by Frisch, that the olfactory sense is located upon the antennæ in bees, and not elsewhere.

Because McIndoo lays so much emphasis upon the effect of the injury of cutting the antennæ upon the normal behavior of insects, Frisch has performed experiments to test its truth. It is generally accepted that male moths are attracted to females by the olfactory sense. After the antennæ were amputated, the males were no longer able to find the females. In some cases the males were deprived of their legs, in others the wings. Such males could not be prevented from finding the females.

Forel, in working upon ants, has found that if the antennæ are cut off, they do not recognize their enemies, but if their legs are cut off, they do. Furthermore, it is a well known fact, according to Frisch, that many important organs, such as the legs, eyes, wings, parts of the head, etc., may be cut without injuring in any way the use of the antennæ.

Methods.

The termites upon which the experiments were performed were found under rocks and old logs in the woods near New

Haven, Conn. For each experiment, a single termite was removed from the main dish and placed by itself in a Syracuse dish which had a piece of black paper pasted on the bottom. The animal was unable to climb over the edge of the dish, and against the black background, could be observed with ease. Both workers and soldiers were experimented upon, but the best results were obtained from experiments upon the soldiers, for these react more definitely to odor stimuli. The large black Camponotus ants were used as controls, since these are known to possess a keen sense of smell, while that of the termite has not heretofore been described in any detail.

Experiments were made in which one or both antennæ were carefully cut off at their bases with iridectomy scissors. Most of the experiments, however, were performed by the application of *local* anaesthetics either to both the antennæ or to the legs. For an anaesthetic, a 1% solution of cocaine hydrochloride was found to give to most satisfactory results. One per cent solution of chloral hydrate and of chloretone were also tried, but they required a longer time in which to become effective and were finally abandoned in favor of the cocaine hydrochloride solution. The anaesthetic was applied with the aid of a fine paint brush. It was somewhat difficult to cover the antennæ without touching other parts of the insect because of their constant activity, but after the first few attempts no trouble was experienced. The antennæ could be caught and held by the moistened brush, and when the brush was removed the drop of the solution remained on the bottom of the dish with the antenna held in it. In this way it was made sure that all parts of the antennæ were equally bathed. In order to be certain that enough of the anaesthetic was applied, the antennæ were treated twice and often three times. The method used in covering the legs was to place a large drop of the anaesthetic between the insect and the metal prong of a pair of forceps upon which the termite had been made to climb. Caught and held in this manner for as long a time as was deemed necessary for the substance to act, the termite was then released. The head of the insect was always held up in the struggle for freedom and so none of the solution ever came in contact with the antennæ. If care was exercised not to put too much of the anaesthetic on, the paralysis would occur in a short time, usually not longer than ten or fifteen minutes, and would wear

off after an hour or two. That the sense organs were actually functionless could be determined by touching the appendages with a needle or forceps. Normal animals react immediately but these partially anaesthetized animals take no notice of such stimulation. Then, too, the effect of the anaesthetic could be seen very often by the difficulty with which the insect walked.

Several different types of odors were used, such as the essential oils of thyme and wintergreen, clove oil, cedar oil, chocolate, onion, beef extract, and a piece of a freshly killed termite. In bringing the odors to the termites, the eye ends of needles dipped in the oils were used. Each odorous substance had to be held within two millimeters of the head of the termite unless the animal was absolutely quiet, a rare occurrence. At least five minutes were allowed to elapse between experiments.

No attempt has been made to keep track of reaction times or to keep detailed records of the type of reactions of a large number of animals, as McIndoo has done, for such experimental work requires a much longer time than could be given. The experiments were meant only to show the presence of an olfactory sense, to find out in a simple way, as far as possible, its probable location, and to try out the method of local anaesthesia, which has never been used to any extent and which if successful, overcomes the point which McIndoo stresses concerning the effect of the shock of the amputation of the antennæ upon the reactions of the animals.

Experiments Upon Termites.

The fact that these insects live underground and feed mainly upon the cellulose of living or dead vegetation, made the choice of odors with which to test them, a somewhat difficult proposition. As in McIndoo's experiments, two of the essential oils, wintergreen and thyme were used, as well as the other oils of cedar and clove. Such substances as chocolate, onion, and beef extract were tried for variety. Onion failed to call forth any response whatever from any of the termites. Chocolate caused a response in one case. Beef extract was "noticed" by one or two of the insects, while the rest paid no more attention to it than if it were some slight obstacle in their pathway. A piece of the body of a freshly killed termite worker gave the best results of any of the odors aside from the oils.

a. Reactions of Normal Termites.

In order to make sure that the insects would not react to any object placed near them, odorous or otherwise, the termites were tested with clean needles or forceps. In most experiments of this sort, no notice of its presence was taken unless the insect perchance happened to bump into it or came into contact with it by accident. In these cases there were immediate reactions due to the tactile stimulations. The workers backed away hastily or stopped, feeling with their antennæ, only to go on again either directly over it or to one side. Occasionally the soldiers would fight it, though most often they would pass by unconcernedly. In a few cases, however, the termites did seem to feel its presence and backed away, as Stokes remarks in his paper. In these instances it was really very difficult to tell whether they backed because of some unknown factor connected with the approach of the object or whether they would have backed anyway in their constant activity. If they did actually feel its approach, there would still be no difficulty in distinguishing their reactions from those due to olfactory stimuli which are much more energetic. The termites which gave the best reactions to odor stimuli paid no attention to these needles.

The reactions of normal termites to the oils of thyme, wintergreen, cedar and clove, were practically the same. All were instantaneous. If the insect was running about the dish as was usually the case, and the needle dipped in the oil was placed two millimeters directly in front, the animal stopped immediately, backed three or four millimeters, and proceeded ahead at another angle. In some cases the reactions with the wintergreen were not quite as speedy as those with the thyme, though with most no difference would be noticed.

The normal workers were not stimulated by chocolate, onion, or beef extract. Only one paid any attention to a piece of a termite body. This one responded only to the extent of waving the antennæ in its direction for a moment, immediately hastening on. Consequently the odor reactions of the workers can only be interpreted by their responses to the oils.

Soldiers, however, were much more satisfactory in their behavior. Their reactions to the oils were the same as those of the workers. They gave no response to onion. Two soldiers

stopped and proceeded in another direction when beef extract was placed near, but this may have been due to some cause other than odor. One soldier only, seemed to respond to the chocolate. This animal, however, was the one which gave by far the best reactions to all odors. This experiment will be discussed in detail later.

(*b*). *Reactions of Termites with Antennæ Removed.*

1. With one or both antennæ cut off.

(a) One antenna of a termite worker was cut off at the base and the animal set aside so that the effect of the operation could be observed. In all respects, the termite appeared to remain normal and did not seem to be at all upset by the mutilation. Its activity was in no way slackened.

(b) When both the antennæ of another termite worker were cut the immediate effect was striking. The animal which had been very active even with one antenna gone, stopped immediately and acted dazed. It found considerable difficulty in walking and its whole body shook spasmodically. Later on, the animal seemed to become more normal though never entirely so.

(c) On another day, three termite workers were removed from the main dish and put into a Syracuse dish. One antenna was cut from one, and both from another, leaving the third as a control. When observed again in two days, they all appeared normal and were tested with a clean needle, oil of thyme and oil of wintergreen. No notice was taken of the clean needle by any of the three. Upon the approach of the wintergreen, the normal termite immediately backed away. The second termite, lacking one antenna, gave a very good though slightly slower reaction. The third termite lacking both antennæ, seemed to notice the odor, but it did not turn away. It merely stopped and worked its mouth parts. The same reactions occurred in each case when the oil of thyme was used. These experiments would indicate that these two essential oils do in some way stimulate the termites in the absence of their antennæ though not as forcibly. Whether they should be called olfactory stimuli is uncertain. Such oils are known to be irritating to the respiratory system as well as to the olfactory organs.

(d) A worker having both antennæ removed and appearing perfectly normal and active, was tested with clove oil. The

animal reacted when the needle was held directly in front. When the needle was moved to the side there was no response whatsoever. This would seem to suggest that the mouth-parts are somewhat sensitive to such stimuli, but this would be natural with irritating oils.

The experiments on termites having their antennæ cut were not as clear as those made on animals whose antennæ were anaesthetized. Such experiments were made only for comparison and because former investigators used this method.

(*c*). *Experiments Using Anaesthetics.*

1. Antennæ treated with chloretone.

(a) Both antennæ of a termite worker were carefully covered with a solution of chloretone, and in an hour and a half the termite acted perfectly normal. There was no response to either thyme or wintergreen. Fifteen minutes later the two oils were again brought near the same animal and there was still no reaction. Even when the antennæ were touched with the needle which had thyme on it, there was no reaction, showing that the antennæ were thoroughly paralyzed.

2. Antennæ treated with chloral hydrate.

(a) Chloral hydrate could not be made to affect the antennæ as thoroughly as the chloretone or the cocaine hydrochloride. Chloral hydrate was applied to the antennæ of a worker and when tested later, the animal reacted to both thyme and wintergreen. More of the anaesthetic was used, but with the same results. When chloral hydrate was placed on the legs, the reaction was equally pronounced.

3. Antennæ or legs treated with cocaine hydrochloride.

(a) In this first experiment the antennæ of a termite worker were twice covered with the cocaine solution. Five minutes after the second application the animal was tested with oil of thyme but showed no response.

(b) The antennæ of a second termite worker were treated with cocaine. Immediately afterwards the animal gave instant reactions to both thyme and wintergreen. Later, when the anaesthetic had had a chance to work and the insect was quieter, it was again tested with thyme. While the oil was held in front of the head there was no reaction, but when it was moved to the side the insect's body swayed away from it.

Five minutes later the same test was repeated and with exactly the same result. The termite acted distinctly irritated, its whole body shaking at intervals. A third test gave the same result. When tested with the oil of wintergreen held in front of the head, no reaction of any sort was apparent. When transferred to the left side, the whole body straightened and the posterior part of the abdomen turned upwards. Such a reaction can hardly be interpreted as anything but an evidence of irritation.

(c) Cocaine was applied to both the antennæ and the legs of a worker. As soon as all the appendages ceased to be sensitive to the touch, the termite was tested with thyme but with no response.

(d) The antennæ of a termite worker were covered with cocaine. They became bent and were directed to each side instead of forward as normally. The animal mouthed them several times. In five minutes there was no reaction to clove oil. Five minutes after this first test, oil of thyme was brought near and there was a slight response. About four hours later the animal was again tested with clove oil but with no reaction.

(e) Once again the antennæ of a worker were treated with cocaine which was applied five times. After an hour the insect became quiet, though it was very ferocious when touched. The antennæ were found to be thoroughly anaesthetized. Upon testing with the thyme in front of the head there was no reaction but when the needle was placed at one side, the termite in a few moments lifted a leg and moved the whole body as if irritated.

(f) Experiments f and g are the most conclusive which have been made and are the ones most to be depended upon. Soldiers were used in both, and since in experiment g the normal soldiers reacted to other odors as well as to the essential oils, the partially anaesthetized ones could be tested with these true odors which are better criteria.

In this experiment two soldiers were placed in separate Syracuse dishes, and their normal reactions to all odors were recorded.

TABLE I.

	1	2
Heat	Animal still throughout; Noticed but did not avoid.	Backed
Wintergreen	Backed	Backed
Thyme	Backed	Backed
Clove Oil	Started back, then jumped at it and bit it	Backed
Chocolate	No response	No response
Onion	No response	No response
Beef extract	No response	No response
Piece of freshly killed termite	No response	No response

After these tests the antennæ of termite (1) were covered with cocaine. When thyme was held in front of the head there was no evidence of any response. When the needle was moved to the side, the termite after a short time raised the legs on that side and the whole body shook. This reaction seemed, as in several previous experiments, to be due to the irritating effect of the oil.

The legs of the second termite were treated with cocaine, the antennæ remaining normal. When tested with thyme the antennæ moved, but there was no backward motion, as in the tests on the normal termites. This was undoubtedly due to the paralysis of the legs.

(g) In this experiment three soldiers were tested, first, for normal reactions, and later, after treatment with cocaine. The first termite of this group gave the most conclusive reactions which have been obtained mainly because it remained quiet throughout the entire experiment.

Termite (1) was the only one in all the experiments which reacted to the odor of chocolate, but the reaction was so clear that it is worthy of a detailed description, especially since this termite gave better responses for all odors than any of the others tested. The piece of chocolate was held near the head of the animal. The antennæ were immediately waved toward it and the insect bit at it furiously. The chocolate was then

TABLE II.

Normal Soldiers	1	2	3
Clean glass rod	No response	No response	No response
Thyme	Instant backing	Instant backing	Instant backing
Wintergreen	Backed	Backed	Backed
Clove oil	Backed	Backed	Backed
Cedar oil	Waved ant. jumped back, snapped mandibles	Backed	Backed
Onion	No response	No response	No response
Chocolate	Good reaction	No reaction	No reaction
Beef extract	Stopped and went in another direction	No reaction	Same as (1)
Freshly killed termite body	Good reaction	Fair reaction	No reaction

moved to the other side of the head and the insect moved its antennæ to that side following the chocolate, again attempting to bite it. When the chocolate was held above, the head was raised and the antennæ waved about.

The reaction of the termite to the piece of the body of the worker was equally interesting. Recognition took place immediately and the animal went around the body twice. By constantly moving the forceps which held it, the termite could be made to follow across the dish. If it was moved at a greater distance than two or three millimeters, the termite lost the odor and went off in another direction. When held above, the soldier raised its head and waved its antennæ. This is the first experiment in which a termite reacted to any odors other than the oils.

After the antennæ were anaesthetized so that they did not respond to the touch and the animal was quiet again, thyme was brought near the head. There was no reaction for 15 seconds. Then the antennæ moved very slightly and the termite started to move ahead. As the thyme was moved to the side the termite moved faster, and when removed altogether, the animal became quiet once more.

With cedar oil, the reaction time was somewhat longer. The soldier tipped its head to the right and lifted the legs on the right side.

With wintergreen, the reaction time was the same as with thyme, only the termite did not start to walk ahead, but only worked its mouth-parts and its legs.

When tested with clove oil, there was a slight reaction seen in the twitching and lifting of the legs on the right side. No working of the mouth-parts took place. When the oil was moved to the right side, the irritation of the legs on that side was emphasized. When transferred to the left side, the legs on that side were raised.

On the other hand, to beef extract, chocolate, and the termite body there were absolutely no responses, though each substance was held on all sides of the insect for a considerable length of time. At the end of the experiment the antennæ were not sensitive to the touch, while the legs were highly so.

The legs of the second soldier of this group were covered with cocaine. After a period of an hour, though the termite had great difficulty in walking, the legs were still slightly sensitive to the touch. There was an immediate reaction to thyme. When tested with the termite body, the soldier followed it as best it could and snapped at it twice. Cocaine was applied four times but the legs continued to remain somewhat sensitive to the touch, though by no means as sensitive as the antennæ which had not been treated. The legs must have been very nearly paralyzed because the termite could hardly walk. Nevertheless, with thyme there was an instant reaction and the response to the termite body was good.

This experiment seems to show that while the legs may remain sensitive to strong irritants, the antennæ alone are the real organs for the distinguishing of odors.

Experiments on Ants.

In order to be certain that the cocaine method is truly reliable, experiments were made upon two large Camponotus ants which are known to have a keen sense of smell. Before applying the anaesthetic, both ants were tested with thyme to which there were instant reactions. The ants bent their abdomens underneath and stroked their antennæ with their fore legs and with their mouth-parts for a space of three

minutes. When tested with wintergreen an equally swift response occurred. During each test the antennæ waved about as though stimulated by the oil. The reaction to the cedar oil was distinct though less violent. Onion, chocolate, and beef extract caused no reactions.

Cocaine was applied to the antennæ of one of the ants. After the antennæ ceased to be sensitive to the touch, the ant was tested with thyme. There was no reaction other than a slight lifting of the legs, such as occurred with the termites. There was no attempt to escape. The thyme was tried several times afterwards, but with no reaction whatsoever. The ant mouthed its antennæ a great deal. Two hours later, the paralysis brought on by the cocaine had passed and the ant reacted normally. This experiment shows that this method is one which can be used for such work to good advantage since it does not cause any injury to the animal yet renders the organ functionless.

Discussion.

When the antennæ of bees are removed by cutting, the insects in almost all cases do not respond to odor stimuli. They appear to behave normally, the only abnormalities being their shortened lifetime and their failure to be stimulated by odors. McIndoo lays all the weight of his argument regarding their failure to respond, upon the injury brought about by the operation. Then when *seven* workers having their antennæ removed, did respond to the three essential oils, after a long reaction period, he concludes that the legs have an olfactory function, regardless of the fact that the odors used were irritating ones. With bees deprived of their antennæ he records no reactions to the stimulation of the true odors such as honey, pollen and flowers. It is hardly fair to draw definite and decisive conclusions from the reactions to the oils. In McIndoo's experiments upon ants, the same criticism may be made. The normal ants were tested with the three essential oils and in addition with the true odors such as honey, combs, pennyroyal, and formic acid, while the mutilated ones were tested only with the three essential oils.

In many of his experiments upon beetles, the insects which had had their antennæ removed were tested only with the three essential oils to which they would naturally respond,

as did the termites, from irritation. When the elytra, wings, and legs were made functionless, the beetles were tested with all the odors and they reacted, though at a much slower rate than normally. Many failed to respond. However, since the animals did respond in some instances, and to true odors, why is it not an indication of the presence of receptors of olfactory stimuli elsewhere—perhaps on the mouth-parts or the antennæ? Yet McIndoo emphatically states that the antennæ have no olfactory function.

When bees' wings are pulled off and their legs covered, the animals respond to the oils and the other true odors, but at a much slower rate. The fact that they do respond, however, seems to indicate that the olfactory sense is not confined to the legs and wings.

Frisch uses only such odors as essence of tube roses or honey. He trains his bees to respond to certain definite odors. Whatever reactions take place after the removal of the antennæ must therefore be quite conclusive in indicating their function as olfactory organs. McIndoo would say that the insects are made abnormal by the operation, yet he himself says that the insects appear perfectly normal in spite of their shortened lives. Since they seem normal, it is reasonable to suppose that their sense of smell would not be interfered with even if they do not live as long. If they were very abnormal, a bee having one entire antenna cut off and all but two segments bearing olfactory organs removed from the other, could never be trained to respond to a new odor.

McIndoo claims that the more segments of the antennæ there are removed the more abnormal the animal becomes. If the abnormality is produced by the severing of certain large sense cells exposing them to the air, why should one cut cause more abnormality than another, that is, if the cells are present the full length of the antennæ? The increase in the abnormality could be more naturally interpreted as being due to the destruction of a greater number of sensory organs, without the use of which the animal becomes abnormal.

McIndoo's description of the pores of the various orders of insects is very accurate and clear, but too much emphasis ought not to be placed upon the structure of any organs when trying to locate a certain sense. It is more reasonable to first locate the sense by careful experiment, as did Frisch, and then

if possible, determine the relation of the organs of that region to that sense.

It would be exceedingly difficult to attempt to correlate the average results of the experiments upon the termites with the anatomy and distribution of the Hicks pores. In the first place, all of these experiments are superficial, and the results are varied. At most they can serve only as a starting point for further systematic investigation. The experiments are sufficient to show, however, that termites do possess an olfactory sense even though it is not very well developed. The average results indicate that the antennæ are distinctly concerned with that sense, since rendering them functionless causes no response to true odors and only the responses to the oils are ones which show irritation. The pores on the antennæ are those of type a and they are few in number, there being but two on each segment and one or two extra ones near the base. Judging from their external appearance, similar pores are present in many places on the mouth-parts. None of these pores are ever found on the legs. None of the Hicks pores were observed on the antennæ. Pores of both types are usually present on some of the mouth-parts. It may be that the pores on the legs are sensitive to chemical stimuli even though not to strictly olfactory ones. The irritated reactions of the termites having their antennæ anaesthetized, when tested with the essential oils, may have been due to the stimulation of these pores, or even to the effect upon the tracheal system.

Though no incontrovertible conclusions can be drawn from the experiments, they promise more conclusive results if similar experiments are carried on in a larger scale. The method of applying local anaesthetics eliminates the difficulties encountered through the injury caused by the operations.

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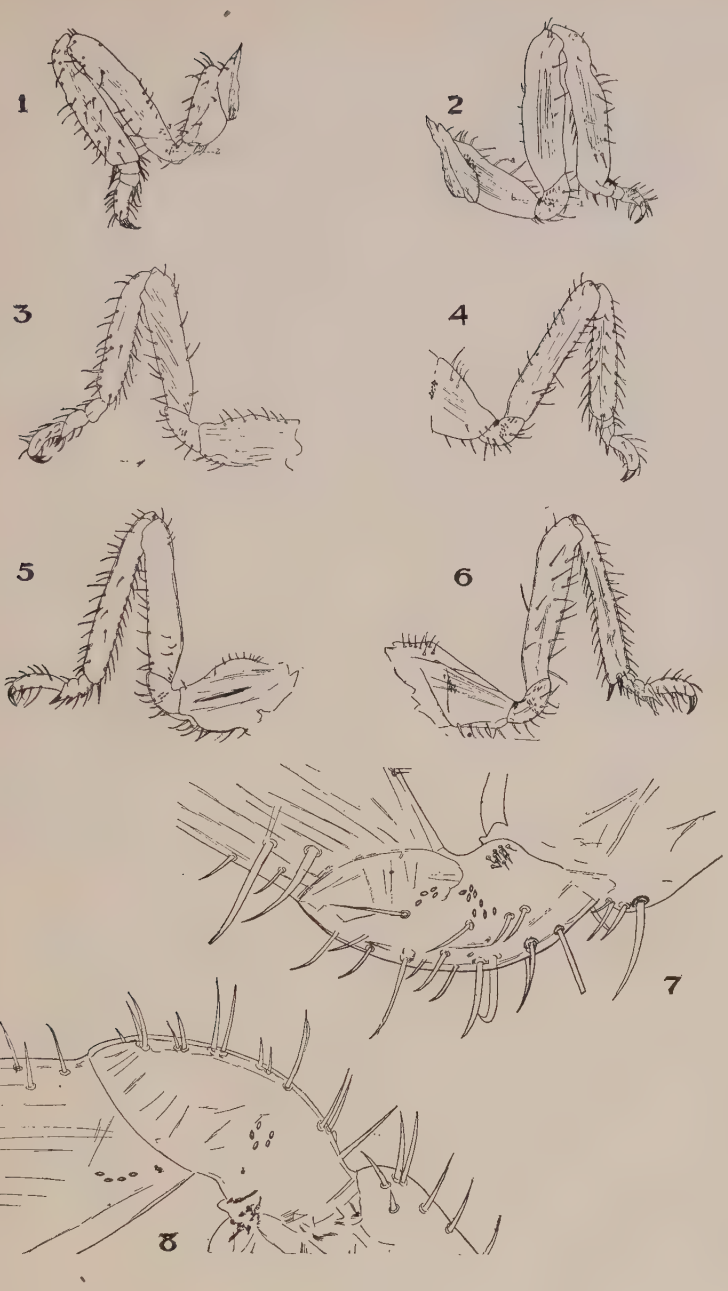
EXPLANATION OF PLATES.

Plate XIX.

Fig. 1. Left front leg of a termite worker, dorsal view showing location of Groups 2 and 3 (× 37).
Fig. 2. Left front leg of a termite worker, ventral view, showing location of the pores of Group 1, and the pegs of Groups a and b, (× 37).
Fig. 3. Left middle leg of the same worker, dorsal view, (× 37).
Fig. 4. Left middle leg, ventral view, (× 37).
Fig. 5. Left hind leg, dorsal view, (× 37).
Fig. 6. Left hind leg, ventral view, (× 37).
Fig. 7. Trochanter of the right hind leg, ventral view, (greatly magnified).
Fig. 8. Trochanter of the right hind leg, dorsal view, (greatly magnified).

Plate XX.

Fig. 9. Dorsal view of right antenna of termite worker, (two segments missing), (× 62).
Fig. 10. Ventral view of left antenna of another termite worker, (× 37).
Fig. 11. Dorsal view of the left mandible of a worker showing the irregularity of the arrangement of the pores and pegs which compose Group 5, (× 37).
Fig. 12. Ventral view of the same mandible showing the pores of Group 6, (× 37).
Fig. 13. Dorsal view of the right mandible, (× 37).
Fig. 14. Ventral view of the same right mandible, (× 37).
Fig. 15. Ventral view of the labrum of a termite worker showing the pores of Group 10, (× 37).
Fig. 16. Dorsal view of the hypopharynx of a worker, (× 37).
Fig. 17. Ventral view of hypopharynx, showing the four Hicks pores of Group 11.
Fig. 18. Dorsal view of the left maxilla of a termite worker, showing the Groups of pegs c_1, c_2, c_3, c_4, and c_5, at the basal portion of each segment, (× 37).
Fig. 19. Ventral view of the left maxilla showing the three Hicks pores which comprise Group 7, and the pores and pegs on the galea, Group 7a.
Fig. 20. Dorsal view of the labium and labial palps of a termite worker, showing the definite row of pores on the galea which make up Group 8, (× 37).
Fig. 21. Ventral view of the labium and labial palps showing the scattered pores of Group 9 and the pegs of Groups d_1, d_2, and d_3, (× 37).
Fig. 22. Longitudinal section through a pore on the antenna of a termite worker showing the innervation and general structure.
Fig. 23. Longitudinal section through a Hicks pore on the trochanter of the leg of a termite soldier.
Fig. 24. Longitudinal section through the pores on the labrum of a termite worker, showing the arrangement of the pores, their innervation, and the flat hairs.



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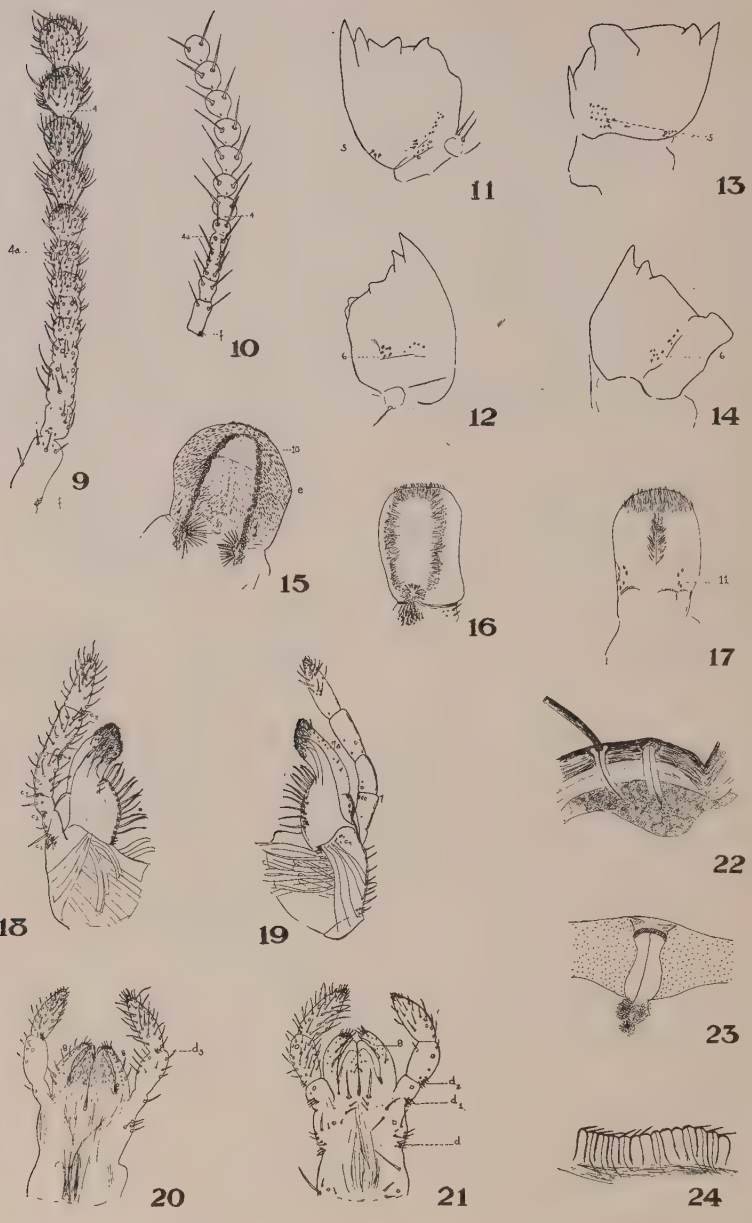

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THE EFFECTS OF THYROID AND SOME OTHER ENDOCRINE PRODUCTS ON PARAMECIUM

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The marked influence of the products of various endocrine glands upon metabolism and growth in the higher animals has, in many cases, been interpreted as indicating a more or less direct and specific effect on cell multiplication. Accordingly, various investigators have turned to unicellular animals as affording a ready means of experimenting directly with single cells; and as a result there are extant several interesting studies on the effects of thyroid and other endocrine products on the Protozoa, in particular on Paramecium.

The first investigation was made in 1908 by Nowikoff (1) who, while studying the chemotactic reactions of Paramecium to thyroid extract, incidentally carried out some experiments to determine the effect of this material on the division rate, and concluded: "Die Resultate des Experimentes sind insofern überraschend, als sie eine ausserst intensive Einwirkung der Schilddrüse auf die Vermehrung der Paramecien zeigen."

The next, as well as the most extensive, studies on the effects of thyroid and other endocrine material on the division rate of Paramecium are those by Shumway. In his first paper (2) he reached the conclusion, from experiments on pedigree lines, that "the effect of thyroid is to increase greatly the rate of division, except at the time when the line is nearing the close of its cycle; and, furthermore, that the effect is not permanent after feeding with thyroid is stopped. Similar treatment with thymus gave negative results." Shumway suggested that the effect of the thyroid is "primarily upon the metabolic activities of the cell;" and that "at the close of the life cycle some element of the protoplasm necessary to digestion is lacking, thus inhibiting the effect of the thyroid." Shumway in his second paper (3) corroborated and extended his earlier conclusions, finding that emulsions of raw thyroids, boiled thyroid, and suspensions of the commercial powder produced a "constant and significant increase of 65 per cent in the rate of division over that observed in the common laboratory medium, hay infusion," the greatest acceleration occurring when the reproductive rate of the control was highest. On the other hand "iodothyrin and iodine failed to produce the thyroid effect," as did likewise "gland suspensions" of thymus, pancreas, spleen, ovary, suprarenal and pituitary body. He

noted that Paramecium ingests and digests particles of the thyroid, and suggested that his results with thyroid feeding are due to the presence of a "remarkably stable hormone in the thyroid which may be classified among the 'dissimilatory' hormones."

Budington and Harvey (4), employing Paramecium and Stylonychia from pedigree cultures, but without daily isolation, tested the effect of thyroid material from animals representing the main classes of vertebrates, since previous workers had employed only mammalian thyroids. They used freshly prepared thyroid glands of fish, frog, turtle, bird and cat, and reached the general conclusion that "thyroid ingredients, no matter from what class of vertebrates the gland be taken, produce essentially the same result when given to ciliate Protozoa as a food or as a factor in the medium in which they live, viz., increased division rate."

Abderhalden and Schiffmann (5) studied the action of various substances, including thyroid material, on the rate of division of Paramecium. From half a dozen experiments they concluded that the rate is markedly increased by the thyroid "opton."

Cori (6) tested the influence of commercial thyroid powder and also thyroxin on the division rate of Paramecium, and stated that "faintly alkaline thyroid extract in hay infusion strongly accelerates" the rate, and that "equivalent concentrations of a solution of thyroxin in hay infusion only very slightly accelerate the rate of multiplication."

Riddle and Torrey (7) gave a summary of experiments with thyroxin on Paramecium, in which they found that the effect of the thyroxin is manifest especially in a slight decrease in the rate of division, and a large increase in excretory activity as indicated by an increased rate of pulsation of the contractile vacuoles and a great decrease in the number of excretory crystals in the endoplasm. Accordingly, they concluded that thyroxin appears to promote katabolic rather than anabolic processes.

Before the publication of the papers by Cori and by Riddle and Torrey we had carried on a series of experiments with various endocrine products and reached the conclusion that "neither thyroxin nor commercial desiccated thyroid nor fresh desiccated thyroid of the turtle produce any significant acceleration of the division rate of Paramecium." This was published in brief form (8). We are glad to note that the conclusions of Riddle and Torrey and our own are apparently in substantial agreement in regard to thyroxin. Together they are opposed to the results of all previous investigators of the problem. Since our preliminary communication was published we have carried on more experiments, and the present paper affords the complete data from the entire study.

Methods. The indication of subtle alterations in the metabolism of a higher animal produced, for instance, by the feeding or the injection of an extract, is obviously complicated by the highly specialized physiological

processes of the animal, as well as by its individual idiosyncrasies. Furthermore, of course, marked differences exist in the action of various substances toward the various tissues as well as toward the same tissue under different conditions. When, however, the organism is reduced to the lowest term, the single cell, these complications are practically eliminated. It is true, to be sure, that the effects produced by extracts, etc., on unicellular forms, may not always afford an exact picture of the effects of these substances upon the specialized cells of the vertebrate. It remains true, however, that a closer approach to the fundamental effects of the substances in question on the basic phenomena of protoplasm should be more readily attained in the Protozoa than in higher forms.

Accordingly, in the present studies, Paramecium was adopted as our "biological reagent" (9) because the ease with which it lends itself to experimental methods makes it one of the most favorable Protozoa for general physiological study, and because we had at our disposal a pedigree race of *Paramecium aurelia* which had been under observation for over fifteen years, and through more than ten thousand generations by cell division (10). This pedigree race has afforded an unfailing supply of Protozoa whose morphology and physiology are accurately known through a series of studies from this laboratory since 1907. Of importance from the standpoint of the problem in hand is the fact that it has been shown that the rate of division of this race is a remarkably accurate indication of the character and temperature of the culture medium; since very slight variations produce significant responses by the organisms. In brief, the reproductive rate may be regarded as essentially a function of the cell's surroundings (11). Furthermore, since all the Paramecia of the race were originally derived from one animal, all the studies were made on "sister" cells, and therefore on the "same protoplasm."

It is necessary to emphasize these facts because it has been shown that the reactions of Paramecia to various substances may be greatly changed by untoward environmental conditions either past or present. In brief, Paramecia pedigreed under conditions which have been demonstrated to be favorable are a *sine qua non* if the animals are to be successfully employed as a "biological reagent."

In preparation for each experiment a single Paramecium was isolated from the main line of the pedigree race with a capillary pipet and placed in a watch glass with a small amount of the regular culture medium, consisting of our standard beef extract. When in the course of a few days the isolated animal had produced by division a sufficient number of cells to start the various lines of the experiment, each animal was isolated on a clean depression slide and supplied either with standard beef extract or with this medium modified with the substance to be tested.

The basic culture medium of beef extract has been the standard medium employed for the culture of Paramecium under consideration and for cultures of other Infusoria in this laboratory since it was formulated after careful experiments made over twelve years ago (12). It has stood the test of time. As usual, for the present experiments a quantity of this solution was made up in water distilled in glass, placed in test tubes and sterilized. Each tube contained sufficient medium for one day's use. Accordingly, the basic culture medium was identical throughout each and every experiment.

The series of depression slides were arranged in moist chambers to prevent evaporation. At the start of the experiment, and also each day, cross-infection was effected, which it is believed rendered the initial daily bacterial flora supplied as food for the Paramecia essentially identical on the various slides. The temperature was maintained at about 23°C., which experiments have shown to be within the optimum zone for this race of Paramecium, but since all the cultures whose division rate is directly compared were carried on synchronously, such slight fluctuations as occurred affected all at the same time and therefore play no significant part in the results.

Every substance in each experiment was tested on four lines of sister cells for ten days. Every day a single animal was isolated from each line, placed on a clean depression slide, and supplied with about 0.3 cc. of fresh medium. At the time of isolation a record was made of the number of divisions which had occurred in each line during the previous twenty-four hours, and these data afford the basis for the results given in the present paper.

In carrying the experiments enthusiastic assistance has been rendered by Miss Hope Spencer of this Laboratory.

Experiment I. The first experiment was planned to show the effect of thyroxin on the division rate of Paramecium. After a number of preliminary tests to determine approximately the greatest concentration of thyroxin which did not too greatly depress the division rate, it was decided to study first the effect of 0.004 per cent and 0.0026 per cent. In view of the fact that thyroxin is practically insoluble in water unless slightly alkaline, NaOH was added: 0.0066 per cent to the former and 0.0044 per cent to the latter. Accordingly, each day sterile beef extract was made up with thyroxin and NaOH in the amounts just stated. In order to determine the effect of NaOH alone and to act as a control for the NaOH necessarily present in the thyroxin solution, there were also made up each day media of standard beef extract with the same percentages of NaOH, viz., with 0.0066 per cent NaOH and with 0.0044 per cent NaOH. Finally, there was the unmodified standard beef extract medium as a control for all.

The definitive experiment extended from November 2 to 11, 1922, and comprised twenty lines of Paramecium: four lines being bred on each of the media described above, which may be referred to as standard beef extract, NaOH (weak), NaOH (strong), thyroxin (weak), and thyroxin (strong). In addition, our basic solution of thyroxin was injected into tadpoles, and its efficacy was proved by markedly accelerating metamorphosis as compared with control tadpoles not so injected.

The following table briefly presents the entire data, giving, in order, the number of divisions in the four lines of Paramecia of each series during the first five-day period; the same during the second five-day period; the total number of divisions for the ten days of the experiment; the number of divisions the thyroxin and the NaOH lines are above or below the standard beef extract control at the end of ten days; and finally, the average daily division rate of the four lines of each culture for the entire ten days of the experiment.

TABLE 1

	DIVISIONS FIRST PERIOD	DIVISIONS SECOND PERIOD	TOTAL DIVISIONS	DIVISIONS ABOVE OR BELOW STANDARD BEEF EXTRACT	AVERAGE DAILY DIVISION RATE FOR 10 DAYS
Standard beef extract	13	14	27		0.67
NaOH, 0.0044 per cent	14	15	29	+2	0.72
NaOH, 0.0066 per cent	16	15	31	+4	0.77
Thyroxin,* 0.0026 per cent	12	4	16	−11	0.40
Thyroxin,* 0.004 per cent	14	1	15	−12	0.37

* In this and the following tables, the percentage of NaOH with the thyroxin has not been indicated since it is the same as in the NaOH controls.

The data in column 5 of table 1 show that both of the NaOH cultures divided slightly more rapidly than the beef control and that both of the thyroxin cultures divided less rapidly than either the NaOH cultures or the beef culture. But long experience with this race of Paramecium has shown that a variation of 0.15 in the average daily rate of division of four lines bred for ten days may be accepted as within the limits of error of the method. Accordingly there is no significant difference between the rates in standard beef extract, NaOH (weak), and NaOH (strong), but there is a significantly lower division rate in both thyroxin media. Accordingly, this experiment indicates in a clear-cut way that the NaOH unavoidably present in the thyroxin media has no appreciable effect, and that 0.0026 per cent and also 0.004 per cent thyroxin in the culture medium produce a significant lowering of the division rate.

Experiment II. This experiment was carried from November 13 to 23, 1922, and is a repetition of the former, except that the concentrations of thyroxin, and therefore of NaOH, employed were lower than in experiment I.

Table 2 gives the data in detail, and shows that 0.0013 per cent and 0.002 per cent solutions of thyroxin produce a significant lowering of the rate of division; the stronger acting as a greater depressant than the weaker solution, and thus agreeing with the results with thyroxin in the former experiment. Furthermore, the data show that 0.0022 per cent and 0.0033 per cent solutions of NaOH produce a lowering of the division rate, the weaker solution producing a very slightly greater effect. Since both the NaOH solutions are weaker than those used in the former experiment, it is evident that these NaOH results do not corroborate those in experiment I. Taking the data from the present experiment alone, one would

TABLE 2

	DIVISIONS FIRST PERIOD	DIVISIONS SECOND PERIOD	TOTAL DIVISIONS	DIVISIONS ABOVE OR BELOW STANDARD BEEF EXTRACT	AVERAGE DAILY DIVISION RATE FOR 10 DAYS
Standard beef extract	24	23	47		1.17
NaOH, 0.0022 per cent	17	13	30	−17	0.75
NaOH, 0.0033 per cent	24	12	36	−11	0.90
Thyroxin, 0.0013 per cent	11	20	31	−16	0.77
Thyroxin, 0.002 per cent	15	4	19	−28	0.47

TABLE 3

	DIVISIONS FIRST PERIOD	DIVISIONS SECOND PERIOD	TOTAL DIVISIONS	DIVISIONS ABOVE OR BELOW STANDARD BEEF EXTRACT	AVERAGE DAILY DIVISION RATE FOR 10 DAYS
Standard beef extract	20	31	51		1.27
NaOH, 0.0011 per cent	15	37	52	+1	1.30
NaOH, 0.00165 per cent	24	27	51	0	1.27
Thyroxin, 0.00065 per cent	21	21	42	−9	1.05
Thyroxin, 0.001 per cent	12	21	33	−18	0.82

be justified in concluding that the depressant effect of thyroxin is due in part to the unavoidable presence in these solutions of NaOH, but it should be emphasized that the strong thyroxin medium, containing the same amount of NaOH as the strong NaOH medium, produced a markedly greater depressant effect than the latter. Obviously, further data are necessary to clear up this point definitely, but the net result from experiments I and II is that thyroxin, in the concentrations tested, lowers the division rate.

Experiment III. From November 27 to December 7, 1922, a third experiment was carried out which is a repetition of the former two, except that the concentrations employed were lower. A survey of the data given

in table 3 corroborates entirely, at lower concentrations, those given in tables 1 and 2 in regard to the depressant effect of thyroxin, and the data in table 1 in regard to NaOH. Thus far, then, the depressant effect produced by NaOH in experiment II remains unique.

Experiment IV. Another repetition of the earlier experiments was carried from December 9 to 19, 1922, but still lower concentrations were used. In addition, lines of Paramecia were tested on standard beef extract

TABLE 4

	DIVISIONS FIRST PERIOD	DIVISIONS SECOND PERIOD	TOTAL DIVISIONS	DIVISIONS ABOVE OR BELOW STANDARD BEEF EXTRACT	AVERAGE DAILY DIVISION RATE FOR 10 DAYS
Standard beef extract	26	26	52		1.30
NaOH, 0.00027 per cent	31	22	53	+1	1.32
NaOH, 0.00041 per cent	31	24	55	+2	1.37
Thyroxin, 0.00016 per cent	30	28	58	+6	1.45
Thyroxin, 0.00025 per cent	27	24	51	−1	1.27
Thyroid	19	11	30	−22	0.75
Pituitary	36	28	64	+12	1.60
Pineal	35	25	60	+10	1.50

TABLE 5

	DIVISIONS FIRST PERIOD	DIVISIONS SECOND PERIOD	TOTAL DIVISIONS	DIVISIONS ABOVE OR BELOW STANDARD BEEF EXTRACT	AVERAGE DAILY DIVISION RATE FOR 10 DAYS
Standard beef extract	18	22	40		1.00
NaOH, 0.000165 per cent	15	19	34	−6	0.85
NaOH, 0.00165 per cent	19	20	39	−1	0.97
Thyroxin, 0.0001 per cent	16	18	34	−6	0.85
Thyroxin, 0.001 per cent	15	14	29	−11	0.72
Thyroid	21	18	39	−1	0.97
Pituitary	18	25	43	+3	1.07
Pineal	20	23	43	+3	1.07

medium to which was added commercial desiccated beef thyroid, pituitary, and pineal material, respectively. The beef thyroid material used in this and subsequent experiments was proved to be highly active in accelerating metamophisis of thyroidectomized and hypophysectomized tadpoles (13).

The complete data are presented in table 4. Thyroxin and NaOH obviously produced no significant effect, such variations as there are from the standard beef extract control being within the limits of experimental error. Accordingly, it appears that we have now reached concentrations just below the threshold of effect. The data in regard to the thyroid

medium show a depression of the division rate, while those from the pituitary and the pineal media show stimulation of the division rate, judged by the standard beef extract control.

Experiment V. This series of eight cultures was bred from February 19 to March 2, 1923. As a whole, it is essentially a repetition of experiment IV, while the first five cultures also constitute a repetition of experiments I, II and III.

TABLE 6

	DIVISIONS FIRST PERIOD	DIVISIONS SECOND PERIOD	TOTAL DIVISIONS	DIVISIONS ABOVE OR BELOW STANDARD BEEF EXTRACT	AVERAGE DAILY DIVISION RATE FOR 10 DAYS
Standard beef extract	20	21	41		1.02
NaOH, 0.00033 per cent	20	14	34	−7	0.85
NaOH, 0.0033 per cent	18	19	37	−4	0.92
Thyroxin, 0.0002 per cent	19	12	31	−10	0.77
Thyroxin, 0.002 per cent	21	14	35	−6	0.87
Pituitary	24	26	50	+9	1.25
Thyroid (commercial)	17	27	44	+3	1.10
Pineal	23	21	44	+3	1.10
Turtle thyroid	31	32	63	+22	1.57
Turtle leg muscle	30	33	63	+22	1.57

TABLE 7

	DIVISIONS FIRST PERIOD	DIVISIONS SECOND PERIOD	TOTAL DIVISIONS	DIVISIONS ABOVE OR BELOW STANDARD BEEF EXTRACT	AVERAGE DAILY DIVISION RATE FOR 10 DAYS
Standard beef extract	16	10	26		0.65
NaOH, 0.00165 per cent	17	13	30	+4	0.75
Thyroxin, 0.0005 per cent	14	13	27	+1	0.67
Thyroxin and pituitary	20	31	51	+25	1.27
Pituitary	24	28	52	+26	1.30
Pineal	24	26	50	+24	1.20

The data show no effect from either 0.00165 per cent or 0.000165 per cent NaOH. Those from thyroxin indicate that this substance, in 0.001 per cent solution, lowers the rate of division, which corroborates the result with the same percentage in experiment III; while in 0.0001 per cent solution it produces no significant effect.

Analysis of the data from standard beef media to which desiccated glandular material has been added shows that neither thyroid, pituitary, or pineal produced any significant effect on the rate of division. This is in striking contrast with the results given in table 4. More data clearly are needed on this point.

Experiment VI. This experiment extended from March 8 to 18, 1923, and consisted of ten cultures. It is practically a repetition of experiment V, though different concentrations of NaOH and thyroxin were tested and two other media were added: freshly desiccated thyroid and leg muscle of the turtle. The turtle thyroid material used in this and subsequent experiments was proved to be highly active in accelerating metamorphosis of thyroidectomized and hypophysectomized tadpoles (13).

Table 6 presents the entire data. It will be noted that both thyroxin and NaOH (strong) did not significantly change the division rate, whereas both thyroxin and NaOH (weak) produced a lowering which should be significant according to our standard. No explanation is apparent for this slightly aberrant result, but it does not obscure the main fact that thyroxin either has no effect or a depressant effect on the division rate. None of the cultures bred in standard beef extract plus commercial desiccated glandular material showed any difference in division rate from that of the standard beef medium, except the pituitary which slightly accelerated the rate. Therefore, the thyroid and pineal data agree with those obtained in experiment V, while the pituitary data agree with those in experiment IV. The series carried on the freshly desiccated turtle thyroid and turtle leg muscle both showed a marked, and, it so happened, an identical accelerating effect on the division rate.

Thus far, therefore, the results with desiccated glandular products in the different experiments show a lack of agreement which is in striking contrast to those from thyroxin and NaOH. But the explanation is not far to seek. The former introduce unavoidable variable factors, the most important of which is changes in the food supply of the Paramecia.

Experiment VII. This series of cultures was conducted from December 28, 1923, to January 7, 1924, and consists essentially of a study of a combination of thyroxin with fresh pituitary material from the frog. Table 7 shows that the results both with thyroxin and with NaOH are concordant with the general results of the previous experiments. Fresh pituitary exactly doubled the rate of division. Thyroxin added to the pituitary medium produced no significant change from that of pituitary alone. The desiccated pineal gland showed a marked acceleration of the rate as compared with the standard beef medium, but no significant difference from the pituitary with or without thyroxin. Obviously, then, the effect of thyroxin in combination with pituitary is not different from thyroxin alone in similar concentrations in standard beef medium. The results from fresh pituitary material agree with those from all the former tests with desiccated material except experiment V; while those from pineal agree thus far only with those of experiment IV. However, it should be noted, neither pituitary nor pineal have ever lowered the rate of division below that of the standard beef control; their action invariably being either neutral or accelerating.

Experiments VIII and IX. These two experiments were carried simultaneously, from January 18 to 28, 1924, not only to supply further data, but chiefly to afford a final demonstration of the precision of the experimental procedure used throughout this study. Obviously, since all the cultures lived synchronously, they were subjected to identical temperature and other environmental conditions, and also the animals in each culture were in the same "physiological condition." Such being the case, the similar cultures in each, as well as the two experiments as a whole, act as reciprocal controls.

TABLE 8

	DIVISIONS FIRST PERIOD	DIVISIONS SECOND PERIOD	TOTAL DIVISIONS	DIVISIONS ABOVE OR BELOW STANDARD BEEF EXTRACT	AVERAGE DAILY DIVISION RATE FOR 10 DAYS
Standard beef extract	24	22	46		1.15
Muscle	33	23	56	+10	1.40
Thyroid	30	28	58	+12	1.45
Pineal	30	24	54	+8	1.35
Pituitary	36	23	59	+13	1.47

TABLE 9

	DIVISIONS FIRST PERIOD	DIVISIONS SECOND PERIOD	TOTAL DIVISIONS	DIVISIONS ABOVE OR BELOW STANDARD BEEF EXTRACT	AVERAGE DAILY DIVISION RATE FOR 10 DAYS
Standard beef extract	27	17	44		1.10
Muscle	34	24	58	+14	1.45
Thyroid	36	22	58	+14	1.45
Pineal	28	22	50	+6	1.25
Pituitary	36	26	62	+18	1.55

Inspection of the data from comparable cultures given in column 5 of table 8 and table 9 discloses that the greatest variation in the average division rate of the four lines for ten days is 0.10, and in one case there is no variation at all. This clearly shows the reliability of the method and that the 0.15 limit of error which we have used in interpreting the data is just.

Studying the same tables from the standpoint of the comparative effects of the material employed, it is apparent that fresh desiccated beef muscle, and commercial desiccated thyroid, pituitary, and pineal material consistently give a slightly higher division rate than the standard beef medium alone, though in one instance the pineal acceleration can not be regarded as significant since it exactly coincides with the limit of error of the method. The thyroid data indicate a slight acceleration of the division rate and so agree only with those from fresh thyroid in experiment VI. The pituitary

data agree with those from all former experiments except experiment V. The pineal data from experiment IX, since they show an acceleration in the rate which is just too small to be significant, and accordingly do not agree with the data synchronously supplied by experiment VIII, afford the sole inconsistency in this final test experiment.

Survey of the results of all the experiments. A synoptic survey of the essential data from all the experiments in regard to thyroxin and NaOH is presented in table 10. From inspection of this table it is evident that of the thirteen cultures tested in regard to sodium hydroxide, ten cultures showed no significant effect, the variation from the standard beef

TABLE 10

	EXPERIMENT I	EXPERIMENT II	EXPERIMENT III	EXPERIMENT IV	EXPERIMENT V	EXPERIMENT VI	EXPERIMENT VII
Sodium hydroxide	0.0044%	0.0022%	0.0011%	0.00027%	0.000165%	0.00033%	0.00165%
	0	—	0	0	0	—	0
Sodium hydroxide	0.0066%	0.0033%	0.00165%	0.00041%	0.00165%	0.0033%	
	0	—	0	0	0	0	
Thyroxin + sodium hydroxide	0.0026%	0.0013%	0.00065%	0.00016%	0.0001%	0.0002%	0.0005%
	0.0044%	0.0022%	0.0011%	0.00027%	0.000165%	0.00033%	0.00165%
	—	—	—	0	0	—	0
Thyroxin + sodium hydroxide	0.004%	0.002%	0.001%	0.00025%	0.001%	0.002%	
	0.0066%	0.0033%	0.00165%	0.00041%	0.00165%	0.0033%	
	—	—	—	0	—	0	

extract control being within the limit of error of the method. Three cultures showed a slight but significant lowering of the division rate. Two of the latter occur in the same experiment (II) and in a solution intermediate in concentration between others which clearly show no effect. Furthermore, the data for the first five days of the experiment (cf. table 2) indicate little or no effect, and only during the second five days is the rate markedly lowered. The remaining culture showing depression was subjected to a very low concentration and was carried synchronously with another culture in experiment VI of much greater concentration which showed no significant effect.

Accordingly, the three cultures indicating that sodium hydroxide (in concentrations present in association with thyroxin) produces a lowering of the division rate are obviously exceptions for which no explanation is apparent. The remaining ten cultures, at widely varying concentrations, definitely establish that the sodium hydroxide necessarily present in the thyroxin cultures has no significant influence, and accordingly that whatever changes in the rate of division the thyroxin cultures reveal, certainly are not attributable to the associated NaOH.

We may turn now to a comparison of the effects of standard beef extract media with and without thyroxin. Inspection of table 10 shows that thirteen cultures were subjected to thyroxin in various concentrations. Eight of these exhibited a lower division rate than that of their respective standard beef extract control. Five of the eight exhibited a lower rate of division than their respective standard beef extract plus sodium hydroxide control. Not a single culture divided at a significantly higher rate than the control.

The lower concentrations of thyroxin, in general, produce no change in the division rate, thus showing that the percentages tested range above and below the threshold of influence of thyroxin under the conditions of the experiment. In brief, the concordant results derived from these experiments afford no support whatsoever for the view that thyroxin accelerates cell division in Paramecium, but, on the contrary, show that, in sufficient concentrations, it depresses cell division in Paramecium.

TABLE 11

	EXPERIMENT IV	EXPERIMENT V	EXPERIMENT VI	EXPERIMENT VII	EXPERIMENT VIII	EXPERIMENT IX
Muscle (beef)					+	+
Muscle (turtle)			+			
Thyroid (commercial)	−	0	0		+	+
Thyroid (turtle)			+			
Pituitary	+	0	+	+	+	+
Pineal	+	0	0	+	+	0

Having shown that thyroxin does not accelerate the rate of division of Paramecium, we may turn to a synoptic view of the data in regard to the effect of desiccated glandular material when added to the standard beef extract medium. In this study there are two unavoidable complications which are not present in the thyroxin and NaOH work, viz., *1*, impossibility of supplying to each culture exactly the same amount of the desiccated material; *2*, alteration of the food supply of the Paramecia, since the glandular material supplies more food for both the Paramecia and for the Bacteria, and therefore the latter multiply more rapidly and afford still more food for the Paramecia. Accordingly, standard beef extract without desiccated glandular material is not an adequate control for beef extract with desiccated glandular material.

However, it is worth while to review the results which are summarized in table 11. Inspection of this table shows that desiccated thyroid (beef commercial and turtle) was tested in six cultures. Three times it produced an acceleration, once a depression, and twice no effect on the division rate as compared with the respective controls on standard beef. Obviously, no general conclusion can be drawn, though, as is to be expected,

the weight of evidence decidedly favors acceleration, when it is borne in mind that the thyroxin content would, if anything, tend to neutralize the acceleration due to increase of food supply of the Paramecia.

A real test of the effect of desiccated thyroid material, however, is afforded when desiccated muscle tissue is employed as a control. Such data are supplied in tables 6, 8 and 9 and show that, in three out of four cultures, thyroid produced no significant alteration in the division rate, and in the fourth culture it produced a lowering of the rate. This result, especially when the details presented in the tables are analysed, shows conclusively that thyroid tissue does not produce a higher division rate than muscle tissue. Or, combining these data with those discussed in the previous paragraph, we may state the same conclusion in another way: using standard beef extract as the control, solutions of desiccated beef tissue accelerate the division rate more than do solutions of desiccated thyroid tissue.

In order to make doubly secure the conclusion that thyroid possesses no stimulating properties peculiar to itself, controls of desiccated pituitary and pineal materials were also employed for the thyroid. Inspection of table 11 shows that both pituitary and pineal invariably either accelerated or produced no effect on the division rate with standard beef as a control. No depression ever resulted. This might be anticipated for the reasons stated above in regard to thyroid. Furthermore, a comparison of tables 4, 5, 6, 8 and 9 shows that of six cultures in which pituitary was a control for thyroid, one indicates acceleration by thyroid, one indicates depression by thyroid, and four indicate no change due to thyroid. Finally, the same tables show that of six cultures for which pineal was a control, two indicate acceleration by thyroid, one indicates depression by thyroid, and three indicate no change due to thyroid.

In brief, the experiments which compare the division rate of animals bred in solutions containing thyroid material with the division rate of animals bred in solutions containing muscle, pituitary, or pineal material, collectively show that thyroid material possesses intrinsically no stimulating property peculiar to itself. Such apparent isolated exceptions to this generalization as appear are clearly explained by the fact that culture media containing thyroid tissue, and likewise those containing muscle, pituitary, or pineal tissue, afford, both directly, and indirectly through bacteria, varying amounts of extra food material.

CONCLUSIONS

No purpose will be served by discussing in detail the experiments of previous workers who have found that thyroid and other endocrine products possess an intrinsic property which stimulates cell division in Paramecium. The crux of the matter, we believe, rests on the character of the controls.

Thyroid and other glandular material supply directly and indirectly more food, and accordingly, for significant results, this factor must be taken into account in devising the controls. It is not clear that previous workers have given this due consideration.

Turning to thyroxin, the generally accepted active principle of the thyroid gland, which has been recently isolated and therefore was not available to the earlier workers, the problem of the control is not so difficult since thyroxin does not alter the food content when added to the basic culture medium. The results of Cori indicate a slight acceleration, though, as he stated, not at all proportional to that induced by thyroid material. Riddle and Torrey, however, obtained depression with thyroxin, and our results accord.

The data supplied by the present study show unequivocally, we believe, that neither thyroid, pineal, nor pituitary material possess intrinsic properties which accelerate cell division in Paramecium. Furthermore, thyroxin does not accelerate cell division in Paramecium. On the contrary, above certain concentrations it depresses cell division in Paramecium.

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REGULATION OF POSTURE IN THE FORE LIMB OF AMBLYSTOMA PUNCTATUM [1]

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THIRTY FIGURES

INTRODUCTION

Previous studies on the transplantation of limbs in the amphibian embryo by Braus ('03, '04, '09), Harrison ('07, '17, '18, '21), and Detwiler ('18, '20) have shown that the limb bud is a self-differentiating system, capable of forming a normal limb even when implanted in a region of the embryo in which normally no limb ever develops. Harrison ('21) found, however, that in certain respects the organic surroundings affect the symmetry relations of the developing limb. Thus, when the dorsoventral axis of the implanted bud is inverted with respect to the corresponding axis of the embryo, the asymmetry of the limb is reversed, i.e., a right limb bud becomes a left limb, whether placed on the right or left side of the body.[2] On the other hand, if the dorsoventral axis is not inverted with respect to the embryo, the prospective asymmetry of the limb bud remains unchanged—a right limb remains a right. The tendency to produce reduplications is also dependent upon the above-mentioned axial relations as well as upon the location of implantation. In a certain group of cases, however, Harrison found that the limb, instead of obeying the above rule, might have its posture regulated by rotation during development. It sometimes regained its normal posture after the bud had been rotated approximately 180°.

[1] Anat. Rec., vol. 21, p. 74.
[2] Harrison ('21), p. 4, rules of symmetry.

It was to study the factors underlying this power of regulation and to analyze further the determination of the axial relations of the limb that the present study was begun.

The first experiments made were transplantations of the limb bud to the dorsal and ventral midlines of the animal. It seemed probable, in accordance with Harrison's work, that a limb bud placed in such a location would give rise to two limbs,[3] since half of the limb bud is placed upon the right side of the body, while the other half is upon the left. This was found to be true in the few cases in which development took place. The results of these experiments were, however, disappointing on account of the failure of the limb to grow properly. Most of the grafts placed in these locations undergo resorption.

In order to investigate further the factors which enter into the development of the limb, transplantation experiments with limb buds have been made in which the following conditions have been varied: 1) The location of the graft; 2) the size of the graft; 3) the amount of rotation; 4) the age of the transplanted tissue.

Experiments have also been done in which the immediate organic surroundings of the limb were rotated.[4] These involve the transplantation of larger areas and the rotation of the limb rudiment within the area. These experiments are as yet incomplete and will be described later.

The present investigation deals with the results obtained by rotating the limb rudiment 90°, 135°, 225°, and 270°. The 180° rotations have been carried out by Harrison ('21). The degree of rotation is measured by the arc through which a given point, say the dorsal end of the dorsoventral diameter, is moved anteriorly around the circumference. The rotation in the case of experiments on the right side is thus measured in a clock-wise direction (figs. 1 and 3).

[3] Wilhelmi ('21) has since performed some ventral implantations.

[4] The rotation of embryonic rudiments has been carried out by Streeter ('09, '14) and Spemann ('06 a, '06 b, '10) in studying the developmental mechanics of the ear vesicle and also by Spemann ('18) for determining the time of differentiation of the parts of the organism.

Two main types of operation were used, viz., transplantation to the location normal to limb development (orthotopic) and transplantation to an unusual environment which has presumably no specific influence upon limb development (heterotopic). The experiments of the first series were constant except for the rotation, but the heterotopic transplants could be varied in size and in the distance of the location of the transplant from the position of the normal limb, which in all of these cases was left intact. These experiments show that the girdle is given off from the limb blastema in the same manner in animals in which the bud has been rotated as it is in unoperated animals, except in cases of reversed asymmetry.

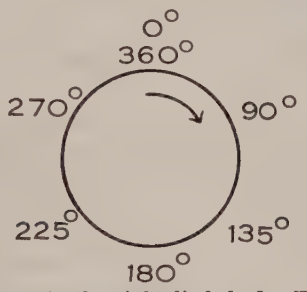


Fig. 1 The circle represents the right limb bud. The arrow shows the direction of the rotation given to the embryonic rudiment, while the degrees express the amount of rotation.

The posture of the resulting limb in the greater majority of cases is regulated in a constant fashion. In the series of 90° and of 270°, regulation is generally in the shortest possible direction for the recovery of posture. The condition of regulation in the other series is complicated by the frequency of reduplication (fig. 2, orthotopic 225° and heterotopic 135°, 225°, and 270°).

Regulation, unless retarded by reduplication, is practically complete in limbs in the normal location. Those which are transplanted to an abnormal location, however, show considerably less rotational recovery. Failure to recover is associated with the size of the girdle. The limbs showing no effect of regulation are found in those series in which the limb was developed in almost complete absence of the girdle.

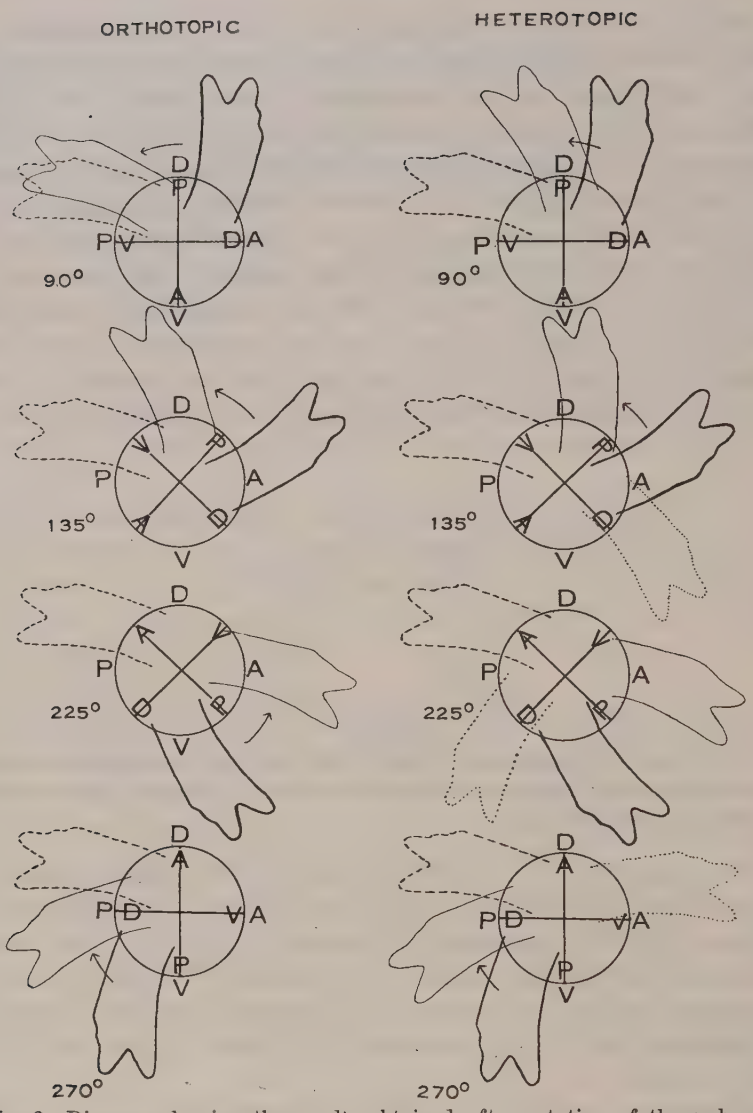


Fig. 2 Diagram showing the results obtained after rotation of the embryonic rudiment. The circles represent the limb bud of the right side, the letters outside the circle representing the cardinal points of the embryo (*D*, dorsal; *V*, ventral; *A*, anterior, and *P*, posterior), while the letters inside the circles represent the cardinal points of the transplanted limb. The broken outlines show the normal posture of the limb, while the heavy outlines show the posture the limb would have assumed if it developed independent of its surroundings. The light outlines show the posture attained by the limb. Reduplicating members are indicated in stippled outline. The arrow indicates the direction of the regulatory rotation.

In the orthotopic location the girdle is a composite structure formed from the parts which have been transplanted and those which have been left within the host. The parts formed from the two components, nevertheless, enter into normal relationship with each other. The girdle of the heterotopic limb is about one-third the size of the normal girdle and is, of course formed entirely out of grafted tissue. Its parts, however, are oriented regularly with reference to the dorsoventral axis of the animal.

This investigation was undertaken at the suggestion of Doctor Harrison, under whose direction it has been carried on. It gives me pleasure to express my gratitude to him for his suggestions and criticisms during its progress.[5]

NORMAL ANATOMY AND DEVELOPMENT

The girdle. A complete account of the development of the parts of the girdle and its form in the adult is found in Detwiler's work ('18, '20), the salient points of which are here given as an aid to the interpretation of the experimental work.

The girdle consists of four portions, the coracoid, procoracoid, scapula, and suprascapula. The process of chondrification proceeds gradually from three centers, one for the scapula, one for the coracoid, and one for the procoracoid, named in the order of their appearance. The suprascapula has no separate center, but chondrifies from the region of the scapula dorsally. In the absence of any connection with the remaining parts of the girdle, the suprascapula, however, chondrifies separately.[6]

Portions of all of the parts forming the girdle are present in the normal limb bud with the exception of the suprascapula. The procoracoid and coracoid are not completely included with the bud, which gives rise to imperfect parts when transplanted elsewhere. Detwiler ('18) found that the normal limb

[5] I am indebted to Miss Lizbeth Krause for redrawing the sketches used in this article.

[6] Control ADP_2 (fig. 25, p. 149).

bud, at the time of the customary stage of operation, stage 29 (Harrison, '18), will give rise to a girdle of about one-third the normal size. Chondrification is completed at about twenty days after the closure of the medullary folds.

The limb. At the time of operation, the limb cannot be called a bud and has nothing by which one can distinguish it except its position. The exact extent of the limb region has been given in the papers of Harrison ('17, '18, '21) and Detwiler ('18, '20) as a circular disc, three and one-half somites in diameter, lying just ventral to the pronephros. This area is located under the third, fourth, fifth, and anterior portion of the sixth somites. Examination of sectioned material does not show definitely the extent of this rudiment. The splanchnopleure and somatopleure are still in contact, and the somatopleure is slightly thickened.

The nomenclature employed by Fürbringer ('72) with reference to the musculature of Salamandra maculosa will be used throughout the following pages. A brief description of the muscles of the shoulder region has been published by Detwiler ('20).[7]

EXPERIMENTAL

1. Materials and methods

The present investigations were carried out on animals varying from stage 27 to stage 30 with a few special cases involving the use of older animals. Specific descriptions covering the procedure in individual series will be given under their respective headings.

The normal procedure followed throughout was to remove a circular disc of three and one-half somites in diameter lying just below the pronephros and centered under the fourth somite. This disc comprising the limb bud was then transplanted to another animal of the same age in which a suitable area had been prepared for the reception of the transplant.

[7] For a more complete description of the internal development and the elements entering into the composition of the girdle and the limb, see Strasser ('79), Lignitz ('97), and Byrnes ('98). Clarke ('78) gives an account of the external development.

At the time of transplantation the limb disc was rotated as shown in figure 3. In considering rotational experiments, it must be borne in mind that the imparted rotation may be varied slightly by healing and also by the subsequent growth of the animal. A definite degree of rotation is regarded as assured when the transplanted tissues have shown very slight changes in the arrangement of the ectoderm as determined by the position of the pigment markings during the first few days after operation.

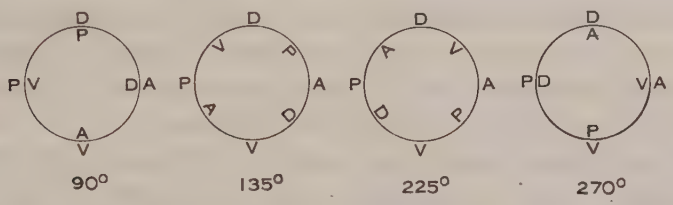


Fig. 3 The circles show the amount of rotation in the several experiments. For lettering see figure 2.

2. Orthotopic 90° rotations (figs. 2 and 3)

a. External development. The transplanted limb becomes evident three to five days after the operation. It is first noted as a slightly rounded swelling with its convexity greater on the dorsal aspect of the bud than elsewhere. This swelling grows dorsally and is inclined away from the body wall. The direction of the limb is fairly constant at this stage and varies only a few degrees in either direction from the transverse plane of the embryo. The typical inclination, however, is seldom equal to the expected 90° deviation from the normal and the few animals in which the grafted limb showed a greater initial deviation from the normal regulated its posture in the same time as other animals in this series. The regulation occurring during early differentiation is very slight.

During the sixth and seventh days, the process of recovery shows several variations which depart markedly from the result to be expected if the limb were self-differentiating in

posture. The angular deviation from the posture expected at this stage shows a much greater variation than in younger larvae. Viewed from the side, or projected upon the sagittal plane, the angle is about 80° from the angle assumed by the normal limb. In addition, the limb is directed away from the body at a considerable angle, usually about 50°, viewed from above. This latter tendency can hardly be said to have any considerable significance, as all transplanted limbs have a tendency to project laterally at a somewhat abnormal angle. The normal limb deviates about 20° or 30° from the horizontal axis when viewed laterally at this stage of development. In a few cases the uptilt of the limb which occurs normally at this stage has been added to the given rotation and the limb is, therefore, directed dorsally and anteriorly.

The eighth and ninth days mark the formation of the first two digits of the operated limb, which is slightly retarded in comparison with the normal limb. While regulation is far from being complete at this stage, it is evident that readjustments of posture are taking place, due to the development of structures at the base of the limb which move it in a direction opposite to that of its rotation at the time of transplantation.

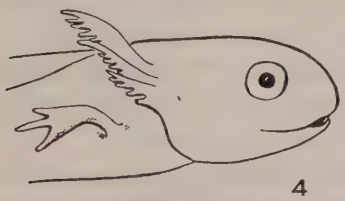
The changes which take place later in development consist in a gradual shifting of posture due to the rotation of the whole limb about its base until nearly regular posture is reached. At the fifteenth day, the limb is generally budding the third digit, the humerus is thrown upward and forward and is drawn toward the side of the body (fig. 4). The formation of the third and fourth digits is generally synchronous with their development in the normal limb and, in this series, the posture has usually become normal by the time this stage is reached (fig. 5).

b. Internal development. The changes occurring within the limb were studied upon preserved material at successive stages in the regulatory process.

At the time of the first appearance of the limb bud upon the surface of the animal, the bud is still a mass of undifferenti-

ated cells. So far as can be observed, development is perfectly regular within the limb. The pronephros is the only structure which shows the effect of the rotation given to the limb disc.[8]

A wedge-shaped mass of cells is slightly separated from the rest of the blastema in an embryo preserved seven days[9] after operation. This mass is destined to give rise to that portion of the girdle which is intrinsic to the transplanted disc. Chondrification begins on the tenth, eleventh, or twelfth day.[10] The animals preserved during this period show irregularities in the distribution of the muscle bundles which will form the ventrolateral sheet of musculature.



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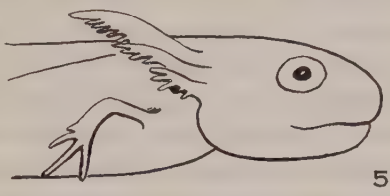

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Fig. 4 Animal DA 55 (90° rotation) twenty days after operation. The limb is practically normal in posture. × 6.

Fig. 5 Animal Da 50 (90° rotation). Regulation complete. × 6.

A complete girdle is formed upon the operated side by the nineteenth day.[11] The suprascapula has joined with the transplanted scapula and is cartilaginous except at its dorsal extremity. The procoracoid and coracoid are also present and have joined to the scapula. There are, however, certain irregularities. The process of the scapula which is continuous with the coracoid is much thicker than normal and the whole of the scapula is thickened. The union of the scapula with the suprascapula is considerably less in extent than normal. All of the muscles connecting the limb to the girdle can be distinguished, but the muscles of the forearm and hand have not yet differentiated from the muscle mass. There is a slight torsion of the forearm and hand, which, however, is probably

[8] DA 71.
[9] DA 41.
[10] DA 75, DA 56, DA 78.
[11] DA 70.

due to flexion and pronation of the arm and not to any defect resulting from the operation. The processus lateralis humeri is developed and the muscles are inserted in their regular order.

Examination of subsequent stages up to that of an animal preserved eighty-four days after operation shows the same consistent course of events. The scapula is thickened considerably transversely, particularly in its more ventral portion. The glenoid cavity is generally regular in form, and when abnormal presents but a slight extension of the dorsal portion of the cartilage. One irregularity which appears frequently is the increase in the size and extent of the suprascapula. The function of the limb in all animals which were kept alive over twenty days was perfect. The slight amount of postural deviation from the normal which remains in very few of the cases was insufficient to disturb function.

The nerves have not been followed to their peripheral endings, but in general they may be said to bear the same relation to the transplanted structure that is shown in the normal limb. The relation of the nerves to the composite scapula is normal and the scapular foramina are in their normal locations.

c. Regulation. Regulation of the posture begins shortly after the limb begins to differentiate from the blastema. For several days after operation, the limb grows as a result of factors which are inherent to the transplanted tissues. This is shown by a certain independence in the direction of growth, whereby the distal portion of the limb, i.e., that portion which first elongates after the superficial appearance of the bud, maintains the direction caused by the original rotation of the limb disc. This growth process is subordinated later by means of the regulating influence which emanates from the basal portion of the limb. At this stage in regulation, the limb is curved slightly so that the distal portion is directed more posteriorly than the proximal portion. Later, with the onset of chondrification in the humerus, the limb straightens and the curved appearance is lost at the same

time that the radius and ulna are formed. Regulation first becomes noticeable when the girdle is being segregated out of the blastema, and the influence of the girdle upon the posture of the limb increases during subsequent development. Table 1, compiled from material preserved on successive days, shows that the posture of the rotated limb undergoes considerable regulation between the eleventh and the fifteenth day of development. It is during this period also that the girdle assumes its definitive form. Complete regulation of posture, however, is not attained until chondrification of the girdle is fairly well completed (table 1, *postural regulation*).

TABLE 1

The conditions found in orthotopic 90° limbs at successive five-day intervals

AGE IN DAYS	CASES	CONDITION OF LIMB			SYMMETRY RELATIONS			POSTURAL REGULATION	
		Single	Redupli-cation	Absent	Normal	Reversed	Inverted	Complete	Incomplete
5 to 10	13	13	0	0	13	0	0	0	13
11 to 15	13	10	1	2	10	0	0	5	5
16 to 20	6	3	0	3	3	0	0	2	1
Over 20 days	22	16	1	5	15	0	0	14	3
Totals	54	42	2	10	41	0	0	21	22

The regulation of the limb takes place by a rotation of the limb as a whole in the reverse direction from the original rotation of the transplanted disc (fig. 6).

While the limb moves as a whole, the girdle is affected by a different process. There are no effects of the operation upon the girdle with the exception of a slight thickening from side to side. The girdle does not undergo rotation as a whole (fig. 6), but when it first appears it has its normal definitive orientation. A turning of this material through an arc of 90° would require a complete reorganization of all structures associated with the girdle, with a consequent change of structural relationships in the later larval forms. This is not found.

The transplanted pronephros was used as a criterion of rotation. In almost every case the lower part of the pronephros was included with the transplanted disc. This retains its original position, i.e., a position which corresponds to its imparted rotation.

The rotation which the limb undergoes in its recovery of normal posture is not caused by a force which acts in a disturbing manner upon the constituents of the limb rudiment. Very few cases show reduplication and the overwhelming majority are regular in both form and function. Of the fifty-

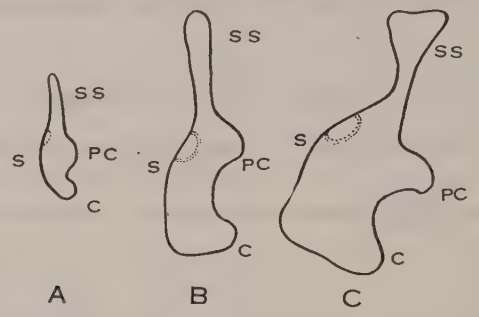


Fig. 6 The girdles produced after 90° rotation in orthotopic series. Animals DA 47 (A), DA 34 (B), and DA 4 (C), preserved, respectively, nine, sixty-seven, and forty-two days after operation. *ss*, suprascapula; *c*, coracoid; *pc*, procoracoid; *s*, scapula. The girdle in each case is complete and is oriented as seen in lateral view. The diagrams were made from camera sketches after dissection. × 18.

four positive cases (table 1), forty-two produced single limbs, while but two showed reduplications. In the majority of cases which were kept alive over twenty days, the regulation of the operated limb is so nearly perfect that it is almost impossible to tell that the animal has undergone operation.

The considerable number of cases of resorption of the limb, shown in table 1, is due to the fact that in the first series of experiments a slightly eccentric area was removed and the pronephros of the right side was left intact, so that the transplant was more ventral in position than normal.[12] All

[12] Transplantation of the limb bud to the ventral and dorsal midlines has been carried out (Anat. Rec., vol. 23, p. 29). In these series, the number of resorptions is extremely high.

cases in which resorption occurred in this series are due probably to the failure of the bud to receive proper blood supply rather than to the initial effect of the operation. In these cases, although the limb has been almost or completely resorbed, the girdles are normally formed with reference to the dorsoventral axis of the animal (fig. 6, *B*).

3. Orthotopic transplantation with 135° rotation (figs. 1, 2, 3)

a. External development. The limb bud appears regularly the third day after operation as an eminence, slightly rounded, with its greater convexity at the anterior portion of the transplant. The swelling increases in size and the limb grows slightly forward, but mostly laterally during the next few days. During this period supernumerary buds may make their appearance and may form any angle with the primary limb.

Resorption of supernumerary parts is frequent, due to the ventral position of the actively growing limb region. Of the eight positive cases preserved in this series (table 2) but two show complete recovery. In these cases, as with the four non-regulated single limbs, there is also a record of resorption of reduplicating members. All cases showed a record of an increased number of digits or abortive attempts at reduplication.

b. Internal development. All cases are analogous to those described in connection with the preceding series of 90° rotation. A complete composite girdle is formed, in which, however, there is an excess of cartilaginous material. All girdles are single and possess but a single fossa for articulation with the humerus.

c. Regulation. The orthotopic limb which has been rotated 135° turns back in the opposite direction from the original rotation (fig. 2). This regulation is not so clearly evident as in the preceding series because of the disharmony between the host and the graft brought about at the time of the operation by the greater degree of rotation. The disharmony shows itself in the larger proportion of reduplications occurring than

in the 90° series. The apex of the limb is drawn posteriorly and projects laterally as the limb is influenced by the development of the girdle. As the portions of the girdle are formed, the basal connection of the limb with the girdle is strengthened and there is a definite postural influence upon the limb. The limb, when single, is finally brought to an approximately normal posture.

4. Orthotopic transplantations with 225° rotation (figs. 2, 3)

a. External development. The limb appears during the fourth or fifth day after operation. It is somewhat behind

TABLE 2

The condition of the limb after rotations of 135° and 225°

SERIES	NUMBER OF CASES	POSITIVE CASES	CONDITION OF LIMB		
			Single	Reduplication	Abortive
Orthotopic 135°	10	8	6	2	2
Heterotopic 135°	18	11	4	7	7
Orthotopic 225°	22	21	6[1]	15	1
Heterotopic 225°	6	3		3	3

[1] Two reversed.

the normal limb in its appearance, but soon overtakes the latter in development. The limb bud shows a marked tendency to reduplicate initially, but this is often followed by the resorption of parts, so that it may be finally reduced to a single member. Four of the six cases of single limbs in table 2 arose through the reduction of reduplicating parts by resorption. Two cases produced limbs which were single throughout their development.

The initial direction of growth in the two cases of single limbs was ventral and slightly anterior. Later in development the limb projected laterally similar to the series with 270° rotation, but with the distal portion of the limb process directed dorsally. The lateral direction of growth persisted

for a longer period than in other series, but the rotation of the limbs in both cases was forward as in the previous series, i.e., in the reverse direction from the operative rotation, so that the limb took up a position approximately 180° from that of a normal limb.

This rotation placed the limb in the position where the reversing effect is at its maximum (Harrison, '21, p. 40) and caused its reversal. When the limb was thus reversed, there was no further rotation toward its prospective normal posture as a right limb, for it had become a left limb upon the right side of the body and had acquired the posture of a left limb.

The four cases in which the limb was reduced by resorption to a single member also showed some regulation of posture. The limb in all of these cases is a right limb, showing, therefore, that no reversal of asymmetry has occurred. Since, however, it is not always possible to ascertain whether the limb which finally remained was the primary or secondary member of the duplex limb, it is necessary to consider the development of these limbs in the light of those cases in which both members of the double appendage persisted.

In fifteen cases reduplicating parts remained after the eighteenth day of development. In the great majority of these cases the limb assumes the posture of a right limb, but bears extra digits and has a thickened and shortened forearm. They have all undergone rotation in the same direction as the operative rotation.

What may happen when rotation of the limb is combined with the process of resorption was shown in those cases which produced single regular limbs by the resorption of one of the buds. In a typical case two distinct buds were present on the fourth day after operation. Both appeared at the same time, the ventral bud growing at first more rapidly than the one which was located slightly dorsal and anterior to it. The two buds were separated by a very slight depression. By the seventh day, the ventral bud, which because of its location and development may be termed the primary (expected) limb

bud, had grown ventrally and slightly anteriorly. The secondary bud had grown dorsally and anteriorly. Seven days after the operation the secondary limb began to outgrow the primary limb, and from this time the primary limb failed to develop properly and degenerated. Development had not yet progressed to a condition in which the asymmetry of the limb could be determined, since the first two digits were just beginning to show when development ceased. This limb was completely resorbed, leaving the secondary limb as the functional member.

The secondary limb rotated just as does a limb which develops from a transplanted disc which has been rotated 90°, i.e., it moved along the arc from 90° to 0° and assumed the posture of a normal right limb. Anatomically, the limb is a right and the girdle likewise. Since this limb is a secondary limb and is a reduplicant of the primary limb, it is clear that the primary limb must have become reversed by virtue of its orientation with regard to the embryo in accordance with the second rule of symmetry.

b. Internal development. All girdles examined were regular composite girdles of the same general type as shown in other orthotopic series. When the asymmetry of the limb was reversed, the girdle was reversed also. In cases with reduced supernumerary appendages, the girdles were clearly girdles of the right side. All the musculature was present and was found to be approximately in its normal location.

c. Regulation. Cases in which the limb develops as a single extremity do not recover their normal posture, but become reversed. The limb rotates as a whole in the reverse direction from the original rotation to a position 180° from normal orientation and becomes a left limb with the posture of a left limb upon the right side of the body. The girdles in these cases show a corresponding reversal of asymmetry.

When reduplication and subsequent resorption of one of the members occurs, as in the case described above, regulation is a secondary one, and it is probably the secondary member of the combination which takes over the function and

posture of the primary. In these cases of secondary regulation, the reduplicating member generally appears in the same location as a limb developing from a disc after a 90° rotation. Further regulation is therefore required to bring the limb into normal position and this is accomplished by rotation through the arc 90° to 0°. Rotation of such limbs may be retarded by the presence of rudiments of other resorbing members and by abnormality in the girdle, but is generally complete.

The degree of disharmony between the graft and the host existing in this series of experiments (225° rotation) accounts for the large number of reduplications. This agrees with Harrison's findings ('21, p. 107).

When both members of a reduplication develop, there is an interadjustment of the two members in the regulation of posture. These limbs rotate, however, as do the transplanted limbs with 270° rotation. The right member rotates through the ventral arc (270° to 360°) and attains approximately normal posture.

5. Orthotopic transplantation with 270° rotation (figs. 2, 3)

a. External development. The limb bud first appears as a definite rounding upon the surface of the animal at stage 39. The bud is less rounded than normal, and during the sixth and seventh days, instead of showing the usual dorsal and slightly posterior direction of growth, grows almost at a right angle to the axis of the embryo so that it is a rounded projection from the body of the animal. This is accentuated during the eighth day, and the close of the ninth day shows a limb which is equal to the normal in development. This is directed ventroposteriorly. The limb is directed about 90° from the normal, which at this stage is about 30° from the horizontal when projected on the median plane of the embryo. The ulnar border, i.e., that portion of the limb bud which would normally give rise to the ulnar border, is at the posterior margin of the limb, the radial border being curved with its greatest convexity at its basal portion. The distal part of the limb points downward and backward. Bifurcation of both the normal and the transplanted limbs is well under way.

The abnormalities associated with the rotated limb make their appearance at this stage. Reduplication or reversal of asymmetry may occur. In general, however, if development proceeds without irregularities, there is a bending of the limbs upon themselves, so that their distal portions are directed downward and backward. The relation of the limb plane to the body wall is abnormal, for the limb maintains proximally a lateral direction of growth and the distal portion bends so as to point in a ventroposterior direction. The limbs come under control of the regulating factor rapidly, and from this time the regulation of the single limbs is a steady rotation of the free extremity as a whole through the short arc 270° to 360° until approximately the normal posture is attained.

There are, however, several other factors which may retard or prevent this regulation. Approximately one-fourth of the animals of this series form reduplications, in which case regulation is retarded. The primary limbs undergo rotation, so that the ulnar border which has up to this time been posterior is now moved dorsally so as to come into a position which approximates the normal, although the limb is still twisted. The distal portions of the limbs during this rotation either grow irregularly with respect to the proximal portions or else become subordinated to the processes of growth occurring at the basal region (fig. 2). The greatest degree of regulation is accomplished when this subordination is complete.

The influence of muscular activity and the chondrification of the girdle begins to affect the posture of the limb at about the sixteenth day. In the majority of cases the limb which has undergone a partial recovery before muscular activity begins is brought into its correct posture by the nineteenth day. In some animals regulation may not be complete until the twentieth day or later, but the conditions of structure are such that very little change can be affected after the twenty-first day.

b. Internal development. Examination of sections of the various stages in the process of recovery shows that conditions are very similar to those occurring in the 90° rotations. At about the fifth day after operation,[13] the scapular portion of the girdle begins to segregate from the limb blastema as a thin layer of cells, which then elongates in a dorsoventral direction, making connection with those parts of the girdle which are inherent to the host. From the sixth to the eighth day[14] there is but little change, the layer of delaminated cells becomes thicker while the limb continues, to some extent, its independence in growth. At about the tenth day,[15] chondrification of the girdle begins, and from this time the free extremity comes more and more under the control of the internal structures.

The musculature, intrinsic and extrinsic, appears at this time as definite collections of muscle fibers, the first to appear being the m. dorsalis scapulae, while the m. pectoralis and m. procoracoideus appear soon after. The m. cucularis is present and may be recognized as a thin band of fibers extending down from the occipital region. Other muscles are still in a very rudimentary condition, although they may be recognized and identified. The muscles are normally formed with reference to the girdle structures and their origins and insertions are approximately normal. The processus lateralis humeri which affords attachment to most of the members of the group first to appear is normal in position.

Parts of the pronephros included in the transplant retain their respective original position with reference to the limb as a whole, and while growth takes place, it is clear that the tubules have not been rotated away from their imparted position.

c. The limbs produced. Table 3 shows the condition of the limb at successive periods of development. The first lot of cases shows that during the period from the fifth to the tenth

[13] DP 48.
[14] DP 49.
[15] DP 52.

day there is little or no visible regulation. The daily records show that there is an increase in the amount of regulation as the age of the animal increases. Of the early animals, two, which have limbs that are still in the blastema stage, must be regarded as indeterminate while eight of the animals, from seven to nine days after operation, show a considerable deviation from the expected inclination and, therefore, may be said to have undergone a partial, although incomplete, regulation.

The regulatory process is more in evidence during the period from the eleventh to the fifteenth day, and most of the

TABLE 3

The conditions found in orthotopic 270° limbs at successive five-day intervals

AGE IN DAYS	CASES	CONDITION OF LIMB			SYMMETRY RELATIONS			POSTURAL REGULATION	
		Single	Reduplication	Absent	Normal	Reversed	Inverted	Complete	Incomplete
5 to 10	15	10	2?	1	9	1	0	0	8[1]
11 to 15	9	8	1	0	7	1	0	5	3
16 to 20	9	8	1	0	8	0	0	6	3
Over 20 days	21	14	6	1	11	2	1	11	3
Totals	54	40	10	2	35	4	1	22	17

[1] Two indeterminate.

reduplications show themselves at this time. Other irregularities are also apparent, such as reduplicating buds. Regulation is complete by the time of feeding, generally twenty-one days after the operation. The animals which were kept for a longer period of time showed no changes in posture.

There are several cases in this group which do not fall into the general group with reference to recovery. The first to be considered are four cases in which the asymmetry of the limb has been reversed.

The normal recovery of posture is accomplished by means of a movement through the shortest possible distance, i.e.,

the arc 270° to 360° (fig. 1). The cases which reverse their asymmetry, however, show that the regulating forces may act in the reverse direction and begin to move the limb as a whole around the greater arc in its recovery. If the dorsoventral axis of the limb is not differentiated at the time that this rotation takes place, the limb is reversed in its asymmetry and undergoes development as would a limb which had been rotated 180° (dorsoventral). If the rotational factor did not act until the dorsoventral axis of the limb had been established, an inverted limb would result, i.e., a limb with the asymmetry of the side of implantation, but upside down with reference to the whole animal. One case occurred in which this condition was found. The record shows that the rotation

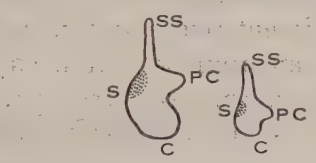


Fig. 7 The types of girdle produced in the orthotopic location after 270° rotation. Lettering as in figure 6. Cases DP 22 and 47. × 18.

of this limb began on the eighth or ninth day, and as a consequence the limb is inverted in its relation to the body as a whole.

d. Types of girdle formed. In general, the type of girdle produced is a regular composite structure made up of the tissues which are contributed by the host and those which are inherent in the transplant. The girdle is quantitatively and qualitatively complete and is oriented normally with respect to the larva (fig. 7).

While this is the general rule, several striking exceptions have been noted. The exceptions are associated with the anomalous limbs described above.

In one case which showed abnormal posture,[16] the girdle appears at first sight to have developed in its orientation as transplanted. The girdle was complete, but was bifid at its

[16] DP 10.

anterior superior margin and there was a process projecting posteriorly. The irregularity of the parts together with the abnormal posture of the limb made diagnosis somewhat difficult. At first sight the parts of the bifid process were thought to be the coracoid and procoracoid and the posterior process the suprascapula. The fallacy of this interpretation was shown by microscopic examination of sections through this region. The chondrification centers show that the parts which grossly resemble the coracoid and procoracoid processes are homologous with the suprascapula, since there is no separate condrification center in this region. The process which extends posteriorly contains two centers of chondrification and is, therefore, homologous with the coracoid and procoracoid processes. The double anterior process is due to the fusion of the suprascapular rudiment remaining in the host with the transplanted girdle and an upgrowth of a small projection from the scapula. The abnormal posture of the limb is due to an abortive reversal of the symmetry relations of the girdle.

The girdle of another case[17] forms an exceedingly irregular example. It is evidently the result of an operation in which the wound was not thoroughly cleaned, the greater part of the girdle-forming structures normal to the site of operation having been left in their original positions. There are two complete glenoid cavities formed, one above the other. Two humeri are present, one of which, judging from the position of the processus lateralis, is the mirror image of the other. The radius and ulna are single, the humeri being fused in the distal third of their extent. The girdle consists of a large sheet of cartilage with divisions anteriorly which mark the position of the normal coracoid and procoracoid. The suprascapular portion is much broader in the anteroposterior direction than is normal, and posteriorly there are several divisions which may be compared with the anterior portions. Just what caused this tremendous hyperplasia of the girdle elements is not clear, especially since there is no regeneration

[17] DP 34.

or restitution incited in this region after the removal of a part in the embryo (Detwiler, '18). The limb is normal distally, except that it possesses one extra digit and it is inverted in posture.

e. Regulation. The regulation of this series takes place by means of rotation of the free extremity as a whole through the shorter arc of recovery (fig. 2) to its normal posture in the majority of cases. Rotation is influenced by the development of the girdle as shown by the time of the regulation, and also secondarily by the formation of the musculature which returns the distal portion of the extremity to the normal posture because of the relationship of the musculature to the girdle. Those cases in which regulation is not complete have their explanation in the particular circumstances of their girdle formation.

6. Heterotopic transplantations with 90° rotation (figs. 2, 3)

The following conditions were varied in this series:

1. The location of the transplant with reference to the normal limb in order to ascertain the effect of the transplanted limb upon the posture of the normal limb; to study the interaction between the normal limb and the transplanted (figs. 8 and 9); to isolate the transplanted limb, so that its development and posture were not influenced by the normal limb, and, finally, to find out the effect of the heterotopic girdle upon the regulation of posture.

2. The degree of development of the transplanted limb bud, as compared with that of the host, in order to determine whether the limb would develop in complete absence of girdle and whether there would be regulation of posture in its absence.

3. The size of the transplanted disc in order to study the effect of a very small girdle upon the regulation of posture (figs. 14 and 15).

Most of the operations concern the first variable, involving only a change in the relative positions of the two limbs. Fifty

animals were used in this series, five of which were discarded because of abnormality or death. The experiments are arranged in three groups: *a*) implantations under the 5-6-7th somites; *b*) under the 6-7-8th somites, and, *c*) under the 7-8-9th somites. Several experiments to determine the growth factors posterior to this region were also carried out, but were so nearly uniform in result that they have been classed with those implanted under the 7-8-9th somites.

1. Heterotopic transplantation of the normal limb bud (3½ somites in diameter). *a.* External development. The transplanted limb begins to develop at the same time as the normal limb. The amount of retardation during the early stages is remarkably small. As development progresses, the limb tends to project sharply from the body wall, extending almost laterally at first in its direction of growth and then turning dorsally. The angle which the transplanted limb assumes in its initial posture is almost equal to the angle of rotation imposed upon the limb disc in transplantation, and this persists for a varying period, during which differentiation of the tissues of the limb proceeds. The deviation of the transplanted limb from the normal is much nearer the expected result than in the preceding series. As development continues and the elbow-joint is formed, the limb presents certain peculiarities which require special mention. It is seldom capable of carrying out any but restricted movements. Cases have been noted in which the fore limb has been drawn tightly against the body wall and is incapable of anything except the most limited function. Function is regarded as depending upon whether the limb receives nerves from the normal limb level of the cord, according to Detwiler ('20). The posture of the limb may be nearly normal in its relationship to the body of the larva and still appear anomalous because of lack of pronation.

Reduplications are frequent in this series (table 4) and are chiefly those of the forearm and hand, although in some cases the whole limb is doubled. Regulation of the developing limb by resorption of reduplicating parts frequently occurs.

The number of reduplications appearing in table 4 would be considerably augmented if the material preserved less than fourteen days after operation were to be considered.

The influence of the transplanted limb upon the normal limb is shown in the first set of experiments in this series, in which the transplant was located under the 5-6-7th somites.

TABLE 4

The placement, type, and results of heterotopic operations of 90° rotation[1]

SERIES[3]	NO. OF CASES	POSITIVE CASES	REGULATION[2]	CONDITION OF LIMB		
				Single	Reduplication	Abortive
HDA 5-6-7	16	8	Partially complete	2	3	3 fused with normal
HDA 6-7-8	15	12	Partially complete	3	8	1
HDA 7-8-9	14	9	Partially complete	2	4	3
HDA 38/29	12	12	None	1	8	3
HDA-CPS	3	3	None	0	3	0
Totals	60	44		8	26	10

[1] Only animals over fourteen days of age appear in table.

[2] Regulation is recorded as partial when there has been some rotation of the limb toward the normal posture as very slight, when the limb rotated a little toward its normal posture, and as none when there was no rotation toward the normal posture.

[3] HDA, heterotopic 90° rotation. 5-6-7, 6-7-8, and 7-8-9 represent the numbers of the somites under which the transplant was located. HDA 38/29, a heterotopic transplantation with 90° rotation, using the limb bud of an animal of stage 38 and transplanting to stage 29. HDA-CPS, central portion (1½ somites) of normal limb disc transplanted to a heterotopic location with 90° rotation.

In six of the sixteen cases the transplant fused with the normal limb. The fusion was nearly complete, although in each case certain features were distinguishable in each of the components. When the limbs were so fused, the component which was rotated exerts an influence upon the non-rotated limb and causes it to rotate during its development. The two components always rotate in opposite directions and the amount of this rotation is limited by the degree of fusion.

The interaction of the limbs upon one another can be most clearly shown by the description of a single case.[18] Three days after operation two separate nodules are apparent, the second lying immediately behind the first. The influence of the transplant upon the normal limb was shown by the decided dorsal direction of growth which the normal limb assumed. Fusion was first noted ten days after the operation, at which time the transplant was directed dorsally at an angle of 60° from the horizontal when projected on the median plane of the embryo. The normal limb showed a reduplicating manus which appeared the fourteenth day after operation. Reduplication may be caused in the normal limb by the transplanted limb without fusion (fig. 8).

The rotation which the limb undergoes in attaining its posture is best seen in those limbs which are far enough removed from the normal limb so that there is no fusion of the girdles nor any chance of fusion with the normal limb. This condition is met in those cases in which the limb has been located under the 6-7-8th and 7-8-9th somites. The limbs behave as in the orthotopic series with like rotation and move in the opposite direction from the imparted rotation. The limb posture obtained in these two groups approaches normal except for pronation.

b. Internal development. Microscopic examination of cases of fused limbs shows that generally there are two girdles formed, the first being the complete girdle normal to the host while the second is the transplanted one, a typical heterotopic girdle, consisting of scapula and rudimentary coracoid and procoracoid. The heterotopic girdle is generally fused to the posterior portion of the normal girdle and the procoracoid is nearly obliterated, although the coracoid can easily be recognized. The heads of the humeri are generally separate. The normal girdle is not thickened and the musculature is normally arranged. There is a proliferation of the ventrolateral sheet of musculature which is found in all heterotopic series.

[18] HDA 48.

In cases in which there was no fusion of the limbs or girdles, all parts are easily identified. The heterotopic girdles are normally oriented with reference to the larva (fig. 12).

c. Regulation. The heterotopic limbs regulate by rotating in the reverse direction from the original 90° rotation (fig. 2),

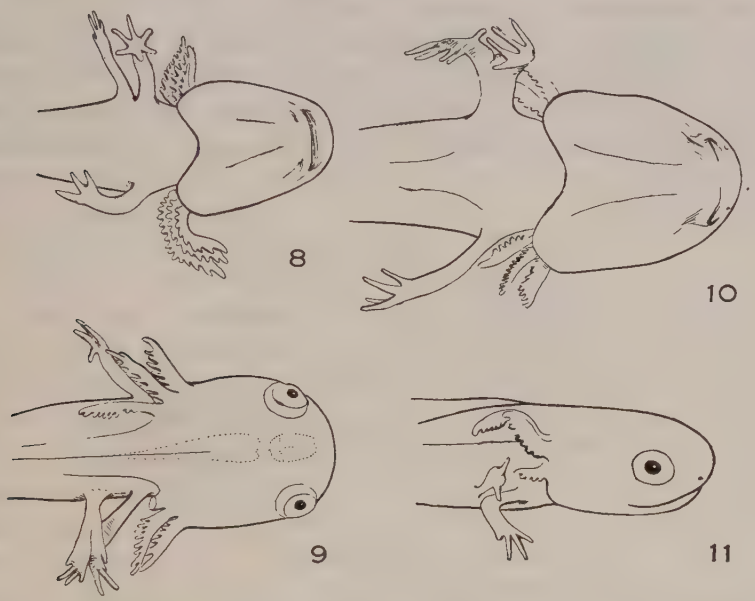


Fig. 8 Animal HDA 54 (90° rotation of heterotopic transplant), twenty-eight days after operation. The transplant has caused reduplication of the normal limb. The transplanted limb has usurped the function of the normal limb and has regulated its posture. × 6.

Fig. 9 Showing the attempt of a reduplicated limb to regulate. There is no pronation, but otherwise the heterotopic limb is regulated in posture. Animal HDA 21 (90° rotation) thirty-four days after operation. × 6.

Fig. 10 Reduplication and fusion of the normal and the transplanted limbs. Animal HDA 50 (90° rotation). Fifty-three days after operation. × 6.

Fig. 11 An abnormal limb with no regulatory recovery. Animal HDA 21 (90° rotation), thirty-four days after operation. × 6.

but regulation is only partially complete. There is, however, a distinct similarity to the regulation of the corresponding orthotopic series. All conditions of the experiment are the same in these two series except for the girdle, which is but one-third the size of the orthotopic girdle, and the location

of the transplant. With the change of location, orthotopic to heterotopic, the extrinsic or myotomic derivatives to the limb are lost.

Rotation in itself is not responsible for the proportion of reduplications occurring in this series as compared with the orthotopic series (cf. tables 1 and 2).

2. Heterotopic transplantations of limbs of an older stage to an embryo of a younger stage of development. The limb bud is removed from an embryo of stage 38 (fig. 13) and transplanted to an embryo in the normal operating stage (stage 29).

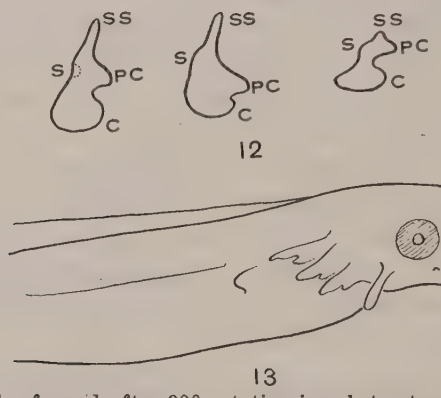


Fig. 12 Girdles formed after 90° rotation in a heterotopic location. Lettering as in figure 6. Cases HDA 17, 43, 28. × 18.

Fig. 13 The appearance of the limb at stage 38. × 9.

Operations upon this older stage require anaesthesia, for which purpose chloretone (0.05 per cent) was used. Since there is a dorsal rounding of the bud at this stage, orientation of the transplant was accomplished without difficulty. The limb so excised was placed upon the flank of the younger embryo in the region of the 7-8-9th somites. The blastema presents a firm mass of tissue which is easily handled and which adheres readily to its new location. It retains its characteristic shape throughout healing and remains in the position in which placed. The limbs which result from this type of operation show great variation in posture, and no

definite rule for development can be outlined (cf. figs. 27 and 28).

While the limbs are topographically complete, marked deficiencies occur in the girdle. The only portion found in any of these cases was a small bit of cartilage probably representing a portion of the scapula.

All of the muscles intrinsic to the limb blastema are present, although functionless not only because of defective innervation, but also because of insufficient surface for muscle attachment. When a cartilaginous structure is present at the base of the limb, the muscles have a tendency to arrange themselves so as to lie in their normal positions. If, however, there is very little or no cartilage present, the muscles which would ordinarily connect the limb to the girdle intermingle with the ventrolateral musculature and are drawn ventrally so that the limb is thrown upward and slightly forward. This movement of the limb is not an effort at regulation, but is caused by the growth of surrounding parts and the intermingling of musculature. It changes the definitive posture of the limb by causing it to migrate, but has no relation to the regulatory recovery occurring in other series. There is no constant relation of the posture of the limb to its imparted rotation or to the normal posture, for there is no structure with which the limb can take up a definite relationship.

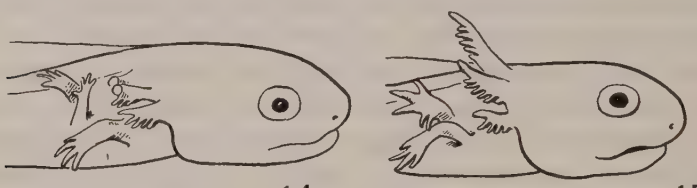
3. Transplantations involving the use of a small disc (1½ somites in diameter). The central portion of the normal limb bud was transplanted to the flank of the embryo dorsal to the pronephric duct. Detwiler ('18) found that this area contained very little of the girdle-forming substances.

Growth of the limb occurs as in the heterotopic limb from the full-sized bud (figs. 14 and 15). There is no retardation of development because of the small size of the transplant. Every case showed reduplication (table 4, HDA-CPS). There is no pronation and the limbs possess no constant postural relation to the organism as a whole.

The limb is complete as the heterotopic limb described above. During development the limb migrates ventrally.

Dissection shows that there is but a small portion of the girdle developed, likely a rudimentary scapula. The girdle, in every case, is covered by the ventrolateral musculature which is interposed between the musculature intrinsic to the bud and the rudimentary girdle.

Summary of heterotopic 90° rotations. The experiments recorded above show that regulation of posture cannot take place when a heterotopic limb is located in close relationship to the normal limb, but that the transplanted limb can cause reduplication and abnormality in the normal limb (figs. 8, 9, and 10), and function can be restricted by the presence of accessory limbs. They also show that, in contrast to the above condition, a limb which has been removed from the



14 15

Figs. 14 and 15 Animals of the series in which but a small area of the normal disc was transplanted. There is no relation of the final postural condition to the 90° rotation of the transplant. This is associated with an almost complete lack of girdle, only a small piece of the scapular rudiment being present. Cases CR-S-DA 1 (fig. 14) and 3 (fig. 15). × 6.

influence of the normal limb will tend to regulate its posture. When there is no girdle or when this is very rudimentary (groups 2 and 3), there is, however, no definite postural regulation.

The group taken as a whole shows that the heterotopic girdle developing from the normal limb bud is of sufficient size to bring about a partial regulation of posture and that when the girdle is smaller than is normal for the heterotopic limb or is absent, regulation does not take place.

7. Heterotopic transplantations with 135° rotation (figs. 2, 3)

Eleven positive cases were secured in this series. All the transplanted limbs but two showed reduplication in some de-

gree, although in two other cases the reduplicating parts were reduced by resorption so as to give a single member.

a. External development. The limb first appears as a rounded swelling posterior and slightly ventral to the normal limb. The limb grows upward and forward and develops at the same time as the normal. Reduplication of the hand occurred in the majority of cases in this series. This, in connection with the posture assumed, makes the interpretation of the asymmetry of the members difficult. Polydactyly is frequent and nearly one-half of the limbs of the series are abortive (table 2, p. 126). Under the head of abortive limbs are placed all cases of resorption and all limbs in which development was noticeably retarded. Four single limbs were produced.

b. Internal development. The same conditions exist here as in the other heterotopic transplantations. A small girdle is formed, the parts of which are normally oriented with respect to the larva. Occasionally the girdle in connection with reduplicating limbs shows hyperplasia, but reduplication of the girdle does not correspond to the degree of reduplication in the limb. The musculature of the limb is arranged regularly with reference to the reduced girdle.

c. Regulation. Owing to the large proportion of cases that reduplicate, the effect of regulation cannot be observed so clearly as in the preceding series. In the two cases of single limbs regulation was partial and the limb rotated back toward normal posture. The other cases also showed rotation and a partial regulation of posture.

d. Comparison with 180° rotations. The limb has been rotated 180° in both the orthotopic and heterotopic locations in Harrison's studies upon the limb. The behavior of limbs in regard to rotational regulation varies in the two cases.

In the orthotopic location, ten cases (non-reversed) of homopleural inverted buds rotated back in the reverse direction from the original rotation, thus regulating their posture. These limbs generally showed at the beginning of their development the effect of the new orientation (Harrison, '21,

fig. 60, p. 41). When the amount of rotation was increased to 190° the limb usually gave rise to duplicities and failed to rotate.

The present work confirms this condition and also the conclusion (op.cit., p. 108) that "in orthotopic grafts the almost completely dominant factor in producing single limbs or reduplications is the harmony or disharmony of the combination."

Harrison found that the heterotopic grafts, however, showed a slight preponderance of reduplications in the harmonic group and a great preponderance of single limbs in the disharmonic group. He also found that the posture of a heterotopic limb is not regulated by rotation after an operative rotation of 180°, but that when the operation places the limb in this position of maximum reversing effect, it is reversed.

When, however, the limb is subjected, as in the present investigation, to rotations which do not bring the limb directly under the influence of the maximum reversing effect, a heterotopic limb will undergo some degree of rotation and will produce a greater number of duplicities than in Harrison's experiments.

8. Heterotopic transplantations with 225° rotation (fig. 2)

Six animals of this type of operation were preserved, in three of which the transplanted limbs showed reduplication, while in the other three the limbs were abortive. The number of cases is too small to permit generalization. None of the limbs showed complete regulation, but all showed some rotation in the same direction as the original rotation. The girdles are regularly formed and are normally oriented in their relationship to the dorsoventral axis of the animal.

9. Heterotopic transplantations with 270° rotation

1. The transplantation of the normal limb bud (3½ somites in diameter). The same general variation in the location of the transplant with reference to the normal limb was followed

here as in the heterotopic series with 90° rotation (p. 135). Since, however, the general results are the same as in the previous series, the cases in this section will be grouped together.

a. External development. The limb appears at approximately the same time as the normal limb. After leaving the condition of the rounded bud (stage 38) (fig. 13, p. 140), it forms a lateral projection from the body wall. The indication of rotation appears five or six days after operation when the bud begins to elongate and becomes flattened distally with its upper surface directed dorsally and slightly posteriorly (fig. 16). The cleft which usually is the expression of normal bifurcation then appears upon the distal margin (figs. 16 and 17). This divides the distal portion of the limb into two parts, each of which may continue development giving rise generally to the simultaneous formation of a primary limb and a reduplicant instead of the first two digits (fig. 18). Under these circumstances, the primary and secondary members are formed synchronously.

When development is slightly delayed the ventral member is the first to appear and the dorsal component, which appears later in development, constitutes the reduplicant. The primary limb at this stage maintains a distinct ventrolateral direction of growth. Fifteen days after operation, the elbow-joint appears. In cases of single limbs, this is formed as in the orthotopic series of the same rotation, the extensor surface being upon the dorsal aspect of the limb. The arm is inclined away from the body, and the forearm appears to be drawn toward the body. Pronation and adduction cannot be carried out. The limb is nearly normal in posture, but the absence of power to adduct and pronate the limb makes it appear to project abnormally from the body of the animal.

b. Internal development. Examination of the girdles shows that the parts are the same as those noted in the preceding heterotopic series. The girdle is the small heterotopic girdle and is regular, in cases of single limbs, in its formation with reference to the dorsoventral axis of the embryo (fig. 26).

Inversion of the girdle does not occur, but occasionally its asymmetry is reversed (fig. 25).

The glenoid cavity may be irregularly formed with reference to the rest of the girdle. This irregularity consists in its flattening, its concavity being directed upward instead of laterally. The rest of the structure is normal in form.

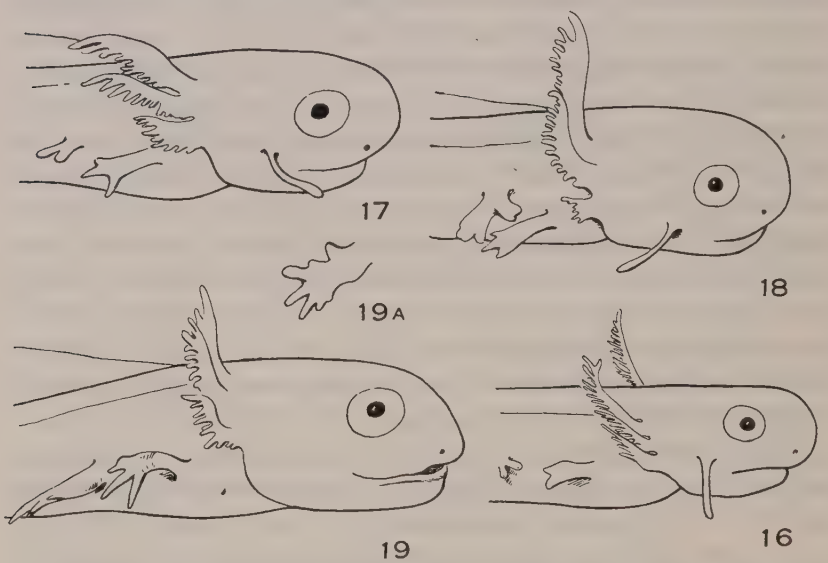


Fig. 16 The early appearance of the limb rotated 270° in the heterotopic location. Animal HDP 29. × 9.

Fig. 17 A later stage than the above. Animal HDP 31 (270° rotation). × 9.

Fig. 18 Lateral view of reduplicating limb of HDP 41 (270° rotation). × 9.

Fig. 19 Lateral view of HDP 27 to show position of limb (270° rotation). × 9.

Fig. 19A Dorsal view of the preceding limb to show reduplicating processes.

Malformation of the glenoid cavity is likely due to the pressure of the head of the humerus upon the scapular rudiment. The anomalous girdles which have been found are due chiefly to overproduction of parts in girdles with reduplicating limbs and represent abortive attempts to reduplicate.

All the muscles intrinsic to the limb bud are present and can be distinguished by their regular position and relations. With the exception of irregularities caused by lack of normal

attachment surface, there is a complete and normal relationship between the musculature and the girdle. There is a proliferation of that part of the ventrolateral musculature which is in the region of the limb, as noted in previous heterotopic series. These muscle processes extend downward intermingling with the limb muscles. The humerus of the heterotopic limb generally shows traces of irregularity in development (figs. 19 and 19 A).

TABLE 5

Results and location of heterotopic transplants with 270° rotation

SERIES [1]	NO. OF CASES	POSITIVE CASES	REGULATION	CONDITION OF LIMB		
				Single	Reduplication	Abortive
HDP 5-6-7	2	1[2]	Incomplete			
HDP 6-7-8	18	15	Incomplete	2	12	1
HDP 7-8-9	21	17	Incomplete	1	16	0
HDP 38/29	10	9	Incomplete	1	7	1
HDP-CPS	5	4	Incomplete	1	2	1
Totals	56	46		5	37	3

[1] HDP, heterotopic transplantation with 270° rotation; 5-6-7, 6-7-8, 7-8-9, are the somites under which the transplant was located; HDP 38/29 = a transplant of the limb bud of stage 38 to an animal of stage 29 with 270° rotation; HDP-CPS = central portion (1½ somites) of normal limb disc transplanted.

[2] Caused reduplication in the normal limb.

c. Regulation. The regulation which takes place in this series is due to the relation of the girdle to the organism and to the normal relationship of the muscles of the limb to the girdle. When the regulation is a secondary one, after reduplication of the limb, the primary member may be more or less completely suppressed and the secondary limb remain. The more ventral component is usually the one to be suppressed (figs. 20, 21, and 24). The remaining limb has its asymmetry reversed and rotates to some extent around the anterior arc.

In cases in which two complete limbs are formed, the ventral one being in harmony with the side of transplantation, the secondary member is a left limb in accord with the rules of symmetry. The two limbs, an harmonic and a disharmonic member, undergo rotation together and the harmonic member attains nearly normal posture by rotating through the shorter arc. In but one case was this condition reversed.[19] Here the limbs moved in the opposite manner from the expected, the harmonic member moving ventrally around the greater arc, while the disharmonic member moved upward and backward. Both limbs were abnormal in posture.

In cases in which there was no attempt to recover posture (figs. 22 and 23), examination of sectioned material shows that a layer of the ventrolateral sheet of musculature overlies the girdle so that the shoulder muscles which developed from the limb bud must have been obstructed as they grew back to their attachments. When the animal turns, flexing or extending the trunk musculature, the limb also undergoes partial flexion or extension. This is not a movement of the limb itself and is to be distinguished from the slight muscular movements which do occur there.

2. Heterotopic transplantations involving the limb of an older animal transplanted to one in a younger stage of development with 270° rotation (fig. 13). The limbs produced in this series show the same behavior as in the corresponding 90° series. There is no relation between the imparted rotation and either the final posture of the transplanted limb or its normal posture (fig. 28).

The girdle is generally very small. In one case a larger mass of tissue than usual was transplanted. From this transplant a girdle of normal heterotopic size developed and the limb nearly recovered its posture by rotating in the arc 270° to 360° as in the preceding series (figs. 27 and 27 A).

3. Heterotopic transplantations with 270° rotation, using a small portion of the limb disc (1½ somites in diameter). The same conditions are found here as in the series of 90° rotation

[19] HDP 43.

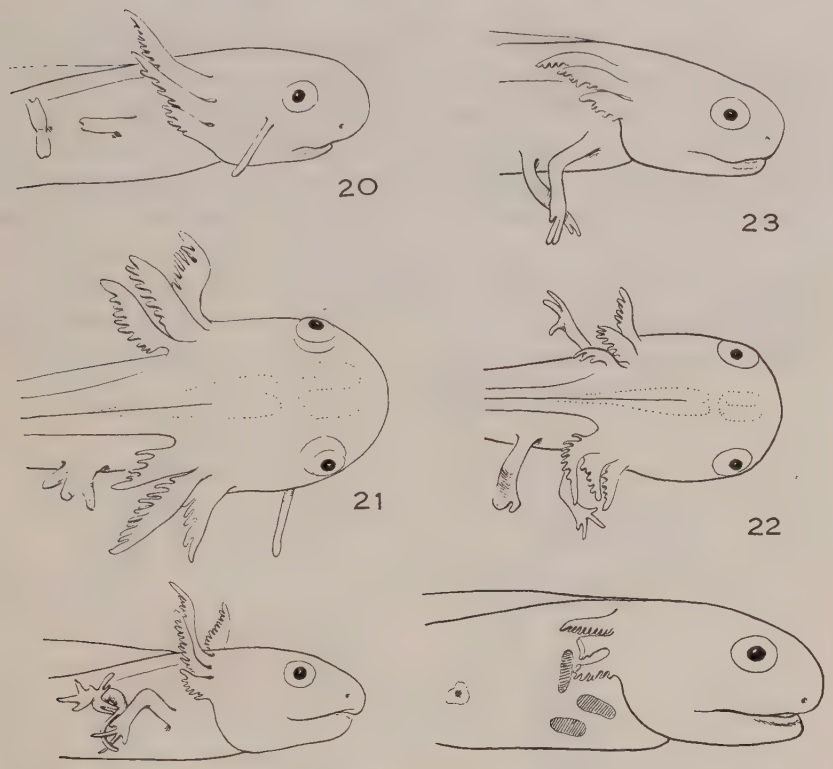


Fig. 20 HDP 43 (270° rotation), ten days after operation. The primary limb is the one which projects dorsally, and is 180° from the expected primary limb. The secondary limb is the one expected as the primary after the rotation which has been imparted to the limb disc. × 9.

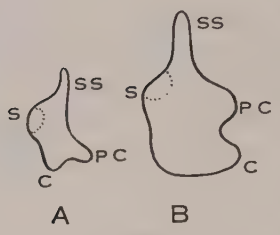
Fig. 21 HDP 43 (270° rotation), fifteen days after operation. There are now three buds, just about 120° apart. The posterior member was the last to appear. × 9.

Fig. 22 HDP 28 (270° rotation), thirty days after operation. This is the posture resulting after the resorption of reduplicating parts. × 6.

Fig. 23 HDP 34 (270° rotation), thirty-eight days after operation; there has been no regulation of posture. There is no contact between the two girdles and there is but a small piece of cartilage at the base of the transplanted limb. × 6.

Fig. 24 HDP 43 (270° rotation), twenty-nine days after operation (figs. 20 and 21). The single ventral filament (*A*) represents the expected limb. The other two buds have fused to form a single limb with dorsal imaging of the hands. The posture resulting is that of a limb and its reduplicant after 90° rotation. × 6.

Fig. 25 ADP 2 (270° rotation). A dissection to show the parts of the girdle developing in the area of operation and also the transplanted girdle which is reversed. Lettering as in figure 6. × 6.



26

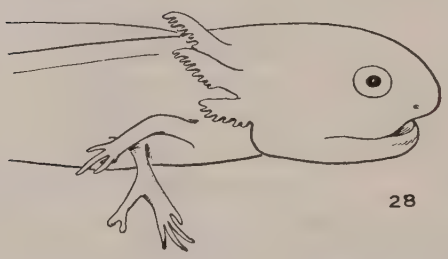


Fig. 26 Girdles formed after 270° rotation in heterotopic location. Orientation is practically normal with reference to the dorsoventral and anteroposterior axes, although the parts are irregular in form (cf. figs. 6 and 12). The girdles are oriented on the page with reference to the cardinal points of the animal. The coracoid is reduced in figure A either through a slight variation of the size of the transplant or by resorption. Animals HDP 19 (*A*) and 15 (*B*). × 18.

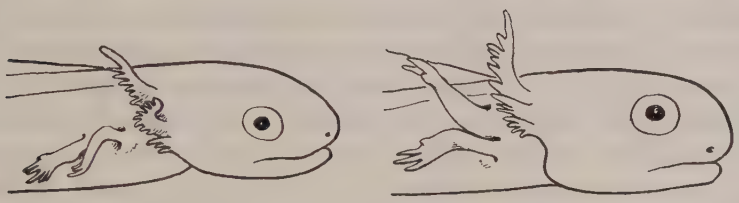
Fig. 27 38/29 HDP 4 (270° rotation), twenty-eight days after operation. The limb has a slight tendency to project downward and the elbow-joint is somewhat anomalous. The limb, however, is single and shows several points of the expected result. × 6.

Fig. 27 A Dorsal view of limb of above animal. × 6.

Fig. 28 38/29 HDP 10 (270° rotation), ninety-two days after operation. This limb never regulated. The original downgrowth was maintained in spite of the reduplication. The animal was kept under observation for a considerable length of time to determine whether any regulation would take place. Dissection showed a small girdle rudiment. × 6.

with corresponding procedure. Practically no girdle is formed, the portion which is so designated being but a small piece of cartilage which sometimes contains a slight groove for the head of the humerus. The growth of the limb is not influenced by the size of the transplanted disc nor is reduplication suppressed in any way. There is no regulation of posture (figs. 29 and 30).

Summary of heterotopic 270° rotations. The heterotopic limb developing from the $3\frac{1}{2}$ somite disc (1) is regulated by rotation in the same direction as the original rotation. This



29 30

Fig. 29 Animal CP-S-DP 3 (270° rotation), thirty-one days after operation, showing lack of regulation of posture in the limbs of this series. × 6.

Fig. 30 Animal CP-S-DP 4 (270° rotation). Neither this animal nor the preceding one shows the presence of any of the girdle structures which can be associated with the normal girdle. × 6.

regulation is less complete than in corresponding orthotopic series and there is a much greater variation in abnormalities and reduplications than in the former (cf. tables 3 and 5).

When development of the girdle is lacking or restricted, as it is in the experiments with older limb buds and with small grafts, then there is no regulation of the limb posture.

DISCUSSION

a. The possible factors influencing the posture of the limb

1. Pronephros. Extirpation of the pronephros has been carried out by Howland ('16, '21) and has also been incident to many of the operations performed by Harrison ('18, '21) and Detwiler ('18, '20).

A small portion of the pronephros was removed with the transplant in most of the cases in the present work and was implanted with the limb disc in its new location. The presence of the pronephros in the transplanted limb is important because of the fact that while the recovery of the limb is taking place, the pronephric tubules remain in their transplanted location and undergo a certain development there. They show, therefore, that the readjustment of the girdle is not a movement of the whole aggregate and that the pronephros has no influence upon the regulation which occurs.

2. The growth of the ventrolateral musculature. The fibers anterior to the limb and those composing the sheet under the limb area are among the first myotomic derivatives to develop. In heterotopic transplants in which development begins late the limb musculature and the ventrolateral musculature are closely united. Either the musculature which would normally appear in the area occupied by the limb is blocked, so that there is an attempt at restitution similar to that noted by Byrnes ('98) and Lewis ('10) after the extirpation of the somites, or there is some attraction exhibited by the girdle or limb musculature which causes an elongation of portions of the ventrolateral sheet.

Absence of the ventrolateral musculature would naturally produce changes which might affect the posture of the limb. The author has been informed by Doctor Detwiler that the removal of the somites results in drawing the limb and girdle further dorsally, but there is no other change in the limb, nor is there any rotation.[20]

3. Deficiencies in the musculature. There are no muscular deficiencies in orthotopic transplantations. All the muscles intrinsic to the limb bud are formed and, in addition, the myotomic derivatives which are present in the host wander down to their normal position and attach themselves regularly to the composite girdle. The heterotopic transplants are also supplied with the intrinsic muscles, the normal attachments of which, however, may be interfered with by the interposition

[20] Detwiler ('18), fig. 8, plate 1.

of the ventrolateral musculature or by the small size of the girdle. In such cases, the recovery of the limb is incomplete and the posture assumed is atypical and irregular. In the absence of the myotomic muscles, processes may be sent down from the somites anterior and posterior to the limb region, but these do no act in the same sense as the missing myotomic derivatives.

4. The girdle parts which are left in the host after the extirpation of the normal limb area (fig. 25, p. 149). As observed by Braus, Harrison, and Detwiler, the parts remaining after the extirpation of the normal limb disc are the suprascapula, the coracoid, and the procoracoid. In all orthotopic operations, these elements united with the transplanted parts, forming a quantitatively complete girdle. This has a normal relationship to the dorsoventral axis of the embryo, although it may be reversed in asymmetry or be irregular in form (figs. 6 and 7).

The heterotopic girdle which develops after a 3½-somite transplantation possesses portions of the coracoid and procoracoid. These are found in their proper orientation with regard to the organism (figs. 12 and 26) and do not show any effect of the operative rotation of the rudiment.

5. The size of the girdle. A complete composite girdle is formed in all orthotopic series. Regulation is more nearly complete and is much more uniform in these than in heterotopic series.

A girdle of one-third the normal size is found in heterotopic operations in which a 3½-somite transplant was used. Regulation of the limb posture is partially complete in these series, i.e., there is a definite tendency for the limb to rotate toward its normal posture, although this may not be completely attained.

The girdle is either rudimentary or absent after two types of operation, that in which an older limb bud is transplanted and that involving grafts of a small size (1½ somites in diameter). In these series there is no regulation of posture.

The size of the girdle is, therefore, the factor which most affects regulation in the limb after its rotation.

b. The regulation of posture as influenced by the degree of rotation

The differences between the results which would be obtained if the limb were an independent differentiating system as regards posture and those obtained after the immediate organic surroundings have exercised their control of posture is shown in figure 2, page 116. It will be noted that posture is most completely regulated in those series in which the operative rotation has placed the limb in the least disharmony with its organic surroundings. So long as the operative rotation is not greater than 180°, recovery, if it occurs, always takes place in the reverse direction from the imparted rotation. At a rotation of 180° the reversing effect of the surrounding parts is at its maximum. When rotation is carried beyond this point a greater proportion of limbs do not recover their posture. There is also a greater number of reduplications (cf. tables 2, 3, and 5). When the limb is rotated 225°, single limbs have their asymmetry reversed, the right bud developing into a left limb and reduplicating limbs may undergo a secondary regulation. After an operative rotation of 270°, the direction of the rotation bringing about regulation is definitely changed, so that the limb moves through the arc 270° to 360° in recovering its posture.

c. Orientation of the girdle

If the girdle were self-differentiating in regard to posture and independent of any influence of the surroundings, one would expect that the part developing from the grafted disc would show the influence of its rotation in its relationship to the organism. Although the result might be clouded by the presence of those parts which develop from the surrounding regions of the host and which are present only in orthotopic transplantations, a definite relationship in the orientation of the parts with reference to the axes of the host would be shown in the heterotopic series (cf. fig. 6, p. 124, and fig. 12, p. 140). Examination of the material shows, however, that

the parts are formed in regular relation to the dorsoventral axis of the embryo. Regulation of axial relations is, therefore, complete in the girdle.

When the limb is a single member and is reversed in asymmetry the girdle is also reversed as found by Harrison ('21) after 180° rotations (fig. 25, p. 149).

d. The interrelationship of the girdle and the limb

The limb has an initial period during which it is practically independent of the surrounding parts of the organism. During this period, the degree of rotation imparted to the limb bud affects the development of the limb as shown by its initial direction of growth. It is during this time that the girdle is in the early process of formation and the scapula is being segregated off from the blastema. The second period of development shows the influence of the girdle which is forming at the base of the limb, and the limb as a whole is brought into harmonious connection with the parts which compose the girdle and the musculature which connects the limb with the girdle.

SUMMARY

1. Limbs rotated 90° recover their postural relation to the organism by means of a rotation of the limb as a whole in the reverse direction from the original rotation. Orthotopic limbs show more complete regulation than do the heterotopic (tables 1 and 4, fig. 2, p. 116).

2. Limbs rotated 135° recover in the same manner. There is a greater proportion of reduplications in this series than in that of 90° (tables 1 and 2).

3. Limbs rotated 225° recover frequently by a secondary regulation and rotate through the dorsal arc in the reverse direction from the original rotation. Reduplications are the rule. Primary single limbs do not recover their posture, but are reversed in asymmetry (table 2).

4. Limbs rotated 270° recover their normal posture usually by a movement of the limb as a whole through the ventral arc

(270° to 360°), i.e., in the same direction as the original rotation. Exceptions to the general rule move in the reverse direction from the original rotation of the limb bud and are either reversed in asymmetry or inverted (tables 3 and 5).

5. The limb, in its recovery, passes through two phases: one, which is independent of associated structures, while the second phase is directly controlled by the girdle (tables 1 and 3).

6. The intrinsic musculature is formed normally in all cases. The muscle attachments are normal, provided there is sufficient girdle to afford attachment surface. Occasionally the limb musculature is mingled with the fibers composing the ventrolateral musculature.

7. Since function is dependent upon innervation from the normal limb level of the cord, few of the heterotopic limbs possess motility in any marked degree. Unmistakable twitching of the limb showed that nervous elements were present. These were also found in sections.

8. Pronation does not occur in the majority of heterotopic limbs. This is because of lack of muscular function, due itself to deficiencies in innervation and not to morphogenetic factors.

9. When the heterotopic limb is placed in close proximity to a normal limb, a partial fusion may result. The transplanted limb may take over the greater function or may cause reduplication in the normal limb (figs. 8 and 10).

10. Irrespective of the rotation given to the limb disc, the girdle is formed in normal relation to the dorsoventral axis of the larva (figs. 6, p. 124; 7, p. 133; 12, p. 140; 25 and 26, pp. 149 and 150). When the limb itself is reversed, the anteroposterior axis of the girdle is also reversed. All cases examined show that the limb and the girdle correspond in their asymmetry.

11. The regulation of posture after rotation is dependent upon the size of the girdle. In heterotopic transplantations where there is an extremely small girdle formed, there is no postural regulation (table 4, HDA-CPS, and table 5, HDP-CPS, figs. 29 and 30, p. 151).

12. After orthotopic transplantation, the scapula and those portions of the rudiments of the coracoid and procoracoid which are located in the transplant unite, respectively, with the suprascapula and the portions of the coracoid present in the host to form a composite girdle. This is quantitatively complete.

13. The formation of the girdle is not associated with a recovery of posture by a movement of the transplanted mass as a whole, for portions of the pronephros transplanted with the limb disc are found in the original location in the transplant. The forces which cause the regulation of the limb posture do not affect the location of the transplanted pronephros.

14. Reduplication may occur after all types of rotation. In the orthotopic rotations, it is most frequent in the 225° series in which the disharmony of the graft is the greatest. This corresponds to the conditions found by Harrison ('21). In the heterotopic series, however, the limb is not regulated by rotation after having been turned through 180° and the number of reduplications is smaller in disharmonic combinations.

15. Resorption may cause the reduction of supernumerary parts, in which case the limb remaining may be either the primary or the secondary member of the combination (figs. 20, 21, and 24, p. 149). The more ventral member of a reduplication is the one more likely to be resorbed.

16. The regulation which occurs in these experiments is not wholly due to functional adaptation for it occurs in heterotopic transplants, which are functionless. It is true that the orthotopic limbs show more complete regulation, but this is due mainly to the normal size of the girdle and only partially to the secondary effects brought about by normal innervation.

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A FURTHER STUDY OF THE EFFECTS OF CONJUGATION AND ENCYSTMENT IN SPATHIDIUM SPATHULA [1]

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THREE FIGURES

It has been conclusively shown that in pedigree cultures of Spathidium spathula under laboratory conditions conjugation produces a significant dynamic effect (Woodruff and Spencer, '21, a, b, '24; Moore, '24). Not only do the majority of exconjugant lines exhibit an initial acceleration in the division rate as compared with the parent non-conjugant lines, but they also attain a greater number of generations. The discovery of endomixis during encystment in the same race of Spathidium (Moore, '24) suggested the possibility of using this form to determine the effect of nuclear reorganization apart from nuclear interchange and synkaryon formation. In Didinium ('15) and Uroleptus ('19), which apparently undergo a process of asexual reorganization in the encysted state, Calkins has found evidence of increased vitality after encystment. Didinium, immediately upon excystation, exhibits a relatively high division rate, which steadily declines until all individuals of the series once more encyst, and Uroleptus, in the two series observed, reveals an initial vitality after encystment as great as that following conjugation.

Throughout the investigations on Spathidium in this laboratory, numerous attempts were made to establish the organism in mass cultures, and several aquarium jars were seeded with

[1] This work was done as Seessel Research Fellow in the Osborn Zoölogical Laboratory, Yale University, 1922–23.

stock individuals. These jars were searched, and from one a Spathidium was isolated October 7, 1922. As Spathidium is fairly uncommon and had not been observed in 'wild cultures' since the discovery of the organism which gave rise to the original culture, it seemed highly probable that this individual belonged to the same race, having passed through the period since the discontinuance of the pedigree culture in the encysted state. There are no means, however, of determining this point conclusively, although in appearance and nuclear constitution individuals of the two cultures are identical.

As soon as conjugation occurred among the descendants of the newly isolated organism, one exconjugant was taken to found a new pedigree culture, which has been carried along from October 11, 1922, to the present time. The usual method of daily isolation on depression slides was employed, with a culture medium of standard beef extract (Woodruff and Baitsell, '11) to which was added a small drop of hay infusion containing Colpidia. An effort was made to keep the room temperature as constant as possible, but fluctuations are inevitable, and throughout a large part of the experiments a thermograph record was kept for comparison with changes in the division rate. Because of the slight uncertainty surrounding the earlier history of this race, it was necessary to confirm the occurrence of nuclear reorganization during encystment and to repeat the previous experiments on exconjugants before considering the physiological effect of endomixis.

EVIDENCE OF NUCLEAR REORGANIZATION DURING ENCYSTMENT

Encystment is of frequent occurrence as the result of starvation, too abundant food, or certain unknown internal processes, and follows the course outlined in a previous paper (Moore, '24) both as to external appearance and nuclear change. The study of more than fifty specimens of encysted and excysted individuals sufficed to show that the reorganization process already described for Spathidium occurs in this race. The frequent coincidence of encystment and conjugation on stock slides complicates the issue to a certain extent,

but the discovery of degenerating macronuclear fragments in early cysts, later cysts with micronuclei only, and excysted individuals with developing or fully formed macronuclear anlagen demonstrates the occurrence of an independent nuclear reorganization. Although it appears highly probable that not all encysted individuals undergo such a process, it has been impossible to distinguish in the living state an accompanying external change characteristic of endomixis. Accordingly, the choice of excysted individuals for further study necessarily involves an element of chance which must be taken into account in any interpretation of results.

EXPERIMENTS

In order to determine the physiological effect, if any, resulting from endomixis during encystment, a comparison of the division rates of parent, exconjugant and excysted cultures was undertaken. According to the usual method, four lines, derived from the first four division products of the exconjugant or excysted individual, were carried along for each series, and the number of generations attained by each recorded daily. The average division rate of all four lines for a period of five days was made the unit of comparison. Since it has already been clearly demonstrated (Woodruff and Spencer, '21 a, '24) that the rejuvenating effect of conjugation is apparent during the first fifteen days as an appreciable acceleration in the division rate of the majority of exconjugant cultures, it was presumed that a like effect resulting from endomixis would be perceptible in the same length of time. Some series were therefore discontinued at the end of three periods, but others were kept for further comparison, since it has been found (Woodruff and Spencer, '21 b, '24) that 80 per cent of the exconjugant cultures attain a greater number of generations than their respective parent cultures during the time both are alive.

HISTORY OF THE MAIN CULTURE, Bb

Before considering the division rates of exconjugant or excysted series it may be well to review briefly the behavior of

the original exconjugant culture from which the others were derived. A graph of its division rate plotted for five-day periods from October 16th to June 7th is shown in the accompanying chart. There is no evidence that conjugation has occurred in the direct line since its origin, and yet the curve shows no progressive decline in vitality. After an abrupt rise during the first three periods, apparently in response to new environmental conditions rather than to the preceding process of syngamy, since the non-conjugant line (A_2) from which Bb was derived showed an equal acceleration, there was a slight decline to an average rate of two divisions per day, which has been more or less maintained to the present time. Periods of temporary depression from which recovery is autonomous form the only departure from a fairly steady rate of fission, and the culture is still flourishing in the 590th generation. A careful search of the records preceding periods of acceleration has completely removed the possibility that an unobserved occurrence of conjugation may have been responsible for the increase. Conjugants do not appear on the daily isolation slides until near the end of the epidemic, after conjugation has been observed for several days on stock slides, and in the majority of cases in these experiments a slight increase in the division rate is recorded before the occurrence of any conjugation.

That periods of depression are in any way concerned with an independent process of nuclear reorganization is improbable, since endomixis has never been observed in Spathidium in the free-living state. Woodruff and Spencer ('24) show no endomixis during vegetative life in the cultures which they studied, and in the present instance, the combined evidence from a number of prepared individuals and from the daily records points to a simple vegetative existence not interrupted by periodic reorganization processes.

Some correlation, however, seems to exist between temperature and rate of fission, which may be modified by quality of food. The daily temperatures as recorded by the thermograph were averaged for five-day periods and plotted along

with the graph of the division rate (fig. 1). From periods 26 to 40 fluctuations in the two curves coincide exactly, and with one exception the fairly even division rate from periods 7 to 26 is accompanied by a similar temperature record. Moreover, in period twelve, where a rise in temperature had no effect upon the division rate, the records show that the 'Colpidia mixture' furnished at the time was unfavorable.

A more striking indication of the injurious effect of improper food conditions is shown by the untimely end of one branch of the main culture, Bb. On April 17th another series of four lines was started from Bb in the 451st generation and subjected to the same cultural methods except for a difference in the 'Colpidia mixture' supplied. The first culture died out May 8th, on the same day as two other series, X_{15} and X_{19}, because of adverse conditions of nutrition. The branch culture, in a more favorable environment, without the intervention of conjugation, continued to divide as before.

THE DIVISION RATE OF EXCONJUGANTS

Throughout the course of the experiment twenty-four exconjugant series were studied. These represent generations F_1 and F_2 in the direct line, and F_1 individuals from excysted lines. In table 1 the division rate of each exconjugant culture is recorded with that of its parent culture for the first fifteen days, the basis of comparison being the number of generations attained by all four lines of a series during that period. Even the use of averages, however, fails to eliminate chance variations incidental to the method of cultivation itself, and in accordance with the plan adopted by Woodruff and Spencer ('24) only a difference of five divisions in three periods is considered a significant variation. Measured by this criterion, twenty of the twenty-four cultures fall within the limits of error and four exhibit a slight increase ranging from 5 to 7.7. These four have varied pedigrees. Two (La and Qa) are F_1 cultures derived from Bb at the 150th and 250th generations, respectively. Oa belongs to the second generation, having been taken at the 207th generation from Ca, which in turn

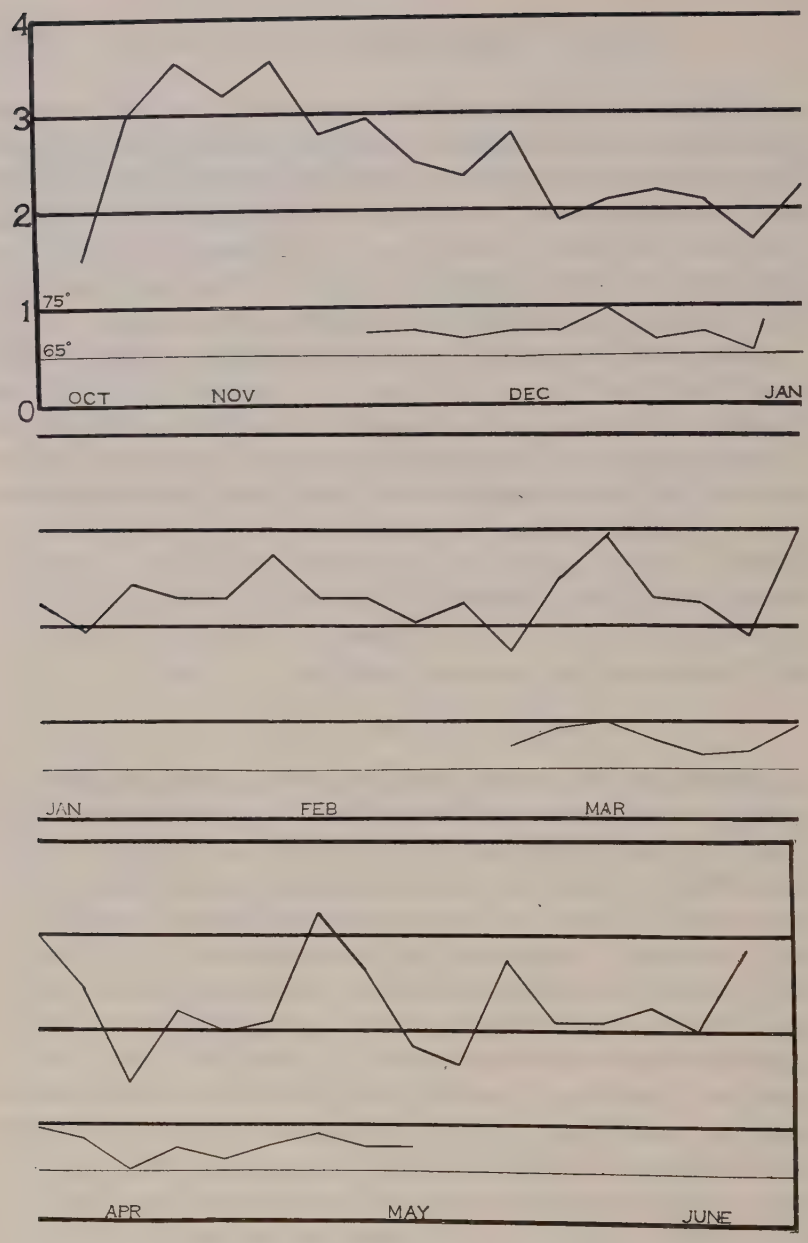

Fig. 1. Graph of the division rate of the culture Bb. Ordinates indicate the average daily division rate of the four lines of the culture, again averaged for each five-day period. Periods are expressed by the abscissa.

At the bottom of the chart is shown a partial temperature record, also averaged for five-days periods, showing a variation from 65° to 75°.

TABLE 1

Division rate of exconjugant cultures—first three periods

SERIES	NUMBER OF GENERATIONS					VARIATION
	I	II	III	Total	Average	
Parent, A_2	21	63	68	152	38.0	...
1. Ba	24	64	74	162	40.5	2.5
2. Bb	30	60	71	161	40.2	2.2
Parent, A_2	32	61	67	160	40.0	...
3. B_1a	36	64	65	165	41.2	1.2
4. B_1b	31	65	75	171	42.7	2.7
Parent, Bb	64	71	56	191	47.7	...
5. Ca	63	66	56	185	46.2	–1.5
Parent, X_1	55	54	49	158	39.5	...
6. Da	43	55	51	149	37.2	2.3
7. Db	48	58	54	160	40.0	0.5
Parent, X_2	55	51	48	154	38.5	...
8. Ea	52	54	50	156	39.0	0.5
Parent, X_3	49	54	48	151	37.7	...
9. Fa	51	54	60	165	41.2	3.5
10. Fb	52	51	54	157	39.2	1.5
Parent, Ca	54	50	47	151	37.7	...
11. Gb	60	52	48	160	40.0	2.3
Parent, X_3	47	20	52	119	29.7	...
12. Ha	52	25	65	142	35.5	5.8
13. Hb	49	25	57	131	32.7	3.0
Parent, Ca	40	26	46	112	28.0	...
14. Ia	41	34	52	127	31.7	3.7
15. Ib	40	35	51	126	31.5	3.5
Parent, Bb	42	44	42	128	32.0	...
16. La	35	42	46	123	30.7	1.3
17. Lb	45	52	55	152	38.0	6.0
Parent, Bb	49	46	46	141	35.2	...
18. Ma	45	49	50	144	36.0	0.8
Parent, Ca	54	38	53	145	36.2	...
19. Na	55	47	55	157	39.2	3.0
Parent, Ca	38	53	37	128	32.0	...
20. Oa	48	61	50	159	39.7	7.7
Parent, Bb	46	55	46	147	36.7	...
21. Pa	34	52	42	128	32.0	–4.7
Parent, Bb	55	46	46	147	36.7	...
22. Qa	62	48	57	167	41.7	5.0
Parent, Bb	33	55	42	130	32.5	...
23. Ra	40	50	36	126	31.5	–1.0
Parent, Bb	51	37	48	136	34.0	...
24. Sa	51	44	49	144	36.9	2.0

came from Bb in the thirty-third generation. Ha is from an excysted culture, X_9, twenty-eight days after its origin. It was in the seventy-fifth generation at the time and had failed to show any increase in division rate over the parent culture. It is interesting to note that in all four cases the records show adverse nutritional conditions during a part of the first three periods which affect the parent cultures more unfavorably than the exconjugants. Oa, which exhibits the greatest acceleration, came from Ca at a time when it was dividing only 1.8 times daily as the result of a sudden change of food. The parent line recovered, but failed to become adjusted to its new conditions as rapidly as the exconjugant, and finally succumbed after the first three periods when the environment once more became unfavorable.

The parent line, Ca, which gave rise to five separate exconjugant cultures, was the only one which died out before its derivative cultures. Its fate may safely be ascribed to external conditions, since two other series were almost lost on the same day. Moreover, its own parent culture, Bb, still survives. This culture (Bb), furthermore, is the original one from which were derived either directly or indirectly all the excysted and exconjugant lines considered in this study except the first four exconjugants shown in table 1. In view of its continued existence without more than temporary loss of vitality, a study of its exconjugants beyond the first three periods has been of little significance. Eleven series were kept from one to twenty-seven periods longer. One outlived its parent culture, four died from environmental causes, and five were deliberately discontinued. One was kept for further comparison with the original culture until June 7th, the last record shown in the chart for the main line. It was then in the 358th generation, having attained exactly the same number of average generations for all four lines as the parent culture during the same thirty periods. Points of slight acceleration and depression, moreover, coincide in every instance in both the parent and exconjugant cultures.

THE DIVISION RATE OF EXCYSTED CULTURES

The successful breeding of the main culture (Bb) of Spathidium spathula under laboratory conditions to the 590th generation has made it impossible to confirm the previous results on the survival value of conjugation in this species (Woodruff and Spencer, '21 b, '24). Through this failure of the criterion for determining the dynamic aspect of nuclear reorganization, whether through amphimixis or endomixis, the proposed study of excysted cultures loses much of its significance. A slight acceleration in the division rate is the utmost that may be expected.

In order to obtain consistent results, excysted individuals were all derived from cysts of the main culture, Bb. Of the fourteen cultures maintained at various times, all but three were started from normally excysting animals. The fully differentiated individuals which gave rise to X_1, X_2, and X_6, however, were obtained by cutting the cyst membrane. In all cases division occurred from one to three days after excystment. Since the period of encystment is relatively short, lasting only from two to five days, it was not considered necessary to take it into account in comparing the division rates of excysted and control cultures. The number of generations attained by each excysted series during the first fifteen days after the establishment of four lines is shown in table 2, with the corresponding division rate of the parent culture.

If a difference of 5 is again taken as the lowest significant variation, it is seen that all but two of the fourteen series fall within the limits of error. These two, moreover, vary only by 5 and 5.3 on the negative side. Not a single excysted series gives evidence of acceleration of the division rate over that of the control. Four excysted individuals not shown in the table died either without dividing or in a very early generation. Three of the twelve cultures which were continued for a longer time came to an end after seven, fourteen, and twenty-two periods, respectively, because of undesirable food conditions, and nine were deliberately discontinued after being

kept from four to fourteen periods without evidence of any physiological effect of encystment. It is true that cultures X_{16} and X_{17} at the end of fourteen periods showed an increase of twelve divisions over the control culture, but after seventy days that is scarcely significant.

TABLE 2

Division rate of excysted cultures—first three periods

SERIES	NUMBER OF GENERATIONS					VARIATION
	I	II	III	Total	Average	
Bb	64	71	56	191	47.7	...
1. X_1	60	64	55	179	44.7	−3.0
2. X_2	66	53	52	171	42.7	−5.0
3. X_3	60	54	63	177	44.2	−3.5
Bb	71	56	59	186	46.5	...
4. X_4	57	54	57	168	42.0	−4.5
5. X_9	62	49	56	167	41.7	−4.8
Bb	58	69	59	186	46.5	...
6. X_5	50	63	52	165	41.2	−5.3
7. X_6	40	72	56	168	42.0	−4.5
Bb	59	50	47	156	39.0	...
8. X_{12}	42	52	50	144	36.0	−3.0
9. X_{14}	46	49	51	146	36.5	−2.5
Bb	46	55	46	147	36.7	...
10. X_{15}	52	52	52	156	39.0	2.3
11. X_{16}	52	55	47	154	38.5	1.8
12. X_{17}	50	59	44	153	38.2	1.5
13. X_{18}	54	53	46	153	38.2	1.5
Bb	44	40	42	126	31.5	...
14. X_{19}	30	43	40	113	28.2	−3.3

DISCUSSION

The disparity in results between these and the far more extensive experiments on exconjugants carried out by Woodruff and Spencer ('24) is not so fundamental as it may at first appear. Although the majority of exconjugant cultures fail to show any marked increase in division rate over the parent cultures, it is equally true that the majority of parent cultures show no progressive decrease in vitality. In the earlier experiments it was found that cultures in which con-

jugation was prevented steadily declined in rate of fission and eventually died out. Only by successive conjugations after more or less definite intervals was it possible to continue the race under laboratory conditions. In these experiments on individuals of the same species—presumably of the same race—the primary condition no longer obtains and there is no a priori reason for anticipating similar results. The main culture, derived originally from an exconjugant, still persists in the 590th generation. Due to the inconstant nature of the 'Colpidia mixture' environmental conditions at times have been such as to produce a high mortality resulting in the sudden loss of several derivative cultures of both exconjugant and excysted individuals, and of one branch of the main line itself. With these exceptions, however, substitution of a more favorable Colpidia culture has thus far been effective in restoring the division rate to normal.

For more than seven months the original exconjugant culture has been maintained in the laboratory without the occurrence of conjugation. It is possible that under wholly satisfactory conditions it might be continued indefinitely without the necessity of syngamy.[2] Should it be conclusively shown, however, that in a proper environment conjugation is not indispensable for the continued life of Spathidium spathula, the fact still remains that in a state of waning vitality conjugation is able to infuse new vigor into a culture, as expressed in an accelerated fission rate, and to prolong its life beyond the limit attained without conjugation (Woodruff and Spencer, '24). The evidence from the great majority of seventy-nine exconjugant cultures is unassailable.

Only twenty-four series derived from exconjugants were studied in the present experiments, and of these only four showed a slight increase in the division rate over their respective parent cultures during the first fifteen days. We have emphasized the fact that the comparatively even division rate of the main culture makes it difficult to test the rejuvenating

[2] For later studies on this culture through lines derived April 14, 1923, cf. Woodruff and Moore, '24.

effect of conjugation and impossible from these experiments to draw any conclusions as to its survival value. Nevertheless, periods of depression do occur, and it is a significant fact that the four cultures which give evidence of accelerated life processes were all derived from the parent cultures which passed through such a period during the first fifteen days. The records in each instance note the occurrence of adverse food conditions which affect the exconjugants as well as the non-conjugants. With the substitution of a more favorable environment, however, recovery is more complete under the added influence of recent conjugation.

The exconjugant culture, Sa, affords another illustration of this fact. On the day that one branch of the main culture died out a conjugating pair was taken from the stock individuals of the same series left from the previous day, and, in a favorable medium, kept for comparison with that branch of Bb which had been fed on a different culture of Colpidia and had shown no loss of vitality. Under exactly the same conditions no difference in the division rates occurred. In other words, that exconjugant, Sa, which outlived its parent culture under adverse circumstances showed no acceleration in division rate over the sister cells of its parent culture which had not been subjected to unfavorable conditions.

In Paramecium it has been shown (Woodruff and Baitsell, '11 b) that, although temperature markedly influences the division rate, yet under practically constant conditions rhythms still persist. In these experiments it is unfortunate that the temperature records are incomplete. Considering only the periods for which they are available, however, it is interesting to note that, although there is a certain irregularity in the division rate during the time when only slight temperature changes are recorded, the periods of acceleration and depression of the division rate are much more pronounced when the temperature shows corresponding fluctuations.

The discovery of endomixis during encystment in this race of Spathidium adds another point to the evidence that it is identical with the race previously studied. Of the fourteen

cultures derived from excysted individuals, however, not one exhibited the slightest appreciable acceleration in the division rate. Two were even somewhat below the parent culture. Whether or not each individual which gave rise to an experimental culture had undergone nuclear reorganization within the cyst is not known. Only the study of sufficiently large numbers of excysted cultures to allow for the element of chance could definitely establish the physiological effect of endomixis during encystment, but with the failure of our criterion the experiments were curtailed. Comparison of exconjugant and excysted lines derived at the same time yields little of value. It may be noted, however, that no excysted cultures are strictly comparable with any of the four exconjugant series which show a noticeable increase. X_{15}, X_{16}, X_{17}, and X_{18} were started at the same time as Oa, which exhibited the greatest acceleration, but not from the same parent. Pa, moreover, derived from Bb at that time, showed no variation in division rate from the parent. These experiments, accordingly, furnish no clue to the problem of the physiological effect of endomixis.

SUMMARY

1. It has been possible to keep a culture of Spathidium spathula under laboratory conditions without conjugation or loss of vitality to the 590th generation.

2. Only a small percentage of exconjugants derived from this culture show a significant acceleration in the division rate during the first fifteen days. In each case where the exconjugant exceeded the parent culture in number of generations both had been subjected to unfavorable environmental conditions.

3. It would appear that, under proper conditions, conjugation in this race is not essential for continued existence, although it does increase the fission rate and prolong the life of declining cultures.

4. No evidence has been obtained as to the physiological effect of endomixis during encystment in this race.

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THE EFFECT OF CHEMICAL COMPOUNDS ON THE PRODUCTION OF THE TETANY SYNDROME

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Many workers have claimed that reactions similar to those resulting from parathyroid extirpation can be secured in certain mammals following administration by one method or another, of various chemical substances. Thus Berkeley and Beebe (2) have secured muscular twitching by intravenous injections of ammonia, which, taken in conjunction with the work of Macallum and Voegtlin (9) who observed that the urine content of ammonia increases in parathyroidectomized dogs, would seem to indicate that ammonia has some significance in the reaction of the animal to parathyroid tetany. Carlson and Jacobson (4), however, find that the increase in ammonia content of the blood of parathyroidectomized dogs falls well within the limit of experimental error. Moreover, Wilson, Stearns and Thurlow (13) have shown that the initial condition during parathyroid tetany is one of low ammonia output and it is only after the onset of tremors and convulsions that the ammonia output is increased.

Paton and Findlay (10) quote Bostock as having found a persistence of tremor after ammonia injection during anesthesia, at which time the tremors of parathyroid tetany are completely suppressed. They have also mentioned their own observations, which seem to show that the very nature of the increased tremor is primarily different. There is a fine tremor after parathyroid extirpation, a coarse, jerky tremor after ammonia injection. The question of ammonia injection as a method of producing parathyroid symptoms would, therefore, seem to be pretty well settled.

Berkeley and Beebe (2) were able to cause convulsions, snapping of jaws and muscle twitchings by the intravenous injection of xanthin. Their results could not be repeated by Voegtlin and Macallum using the same dosage and method of administration.

Biedl (3) suggested that the tetany syndrome might possibly be due to the active principle of ergot. This is not borne out by the work of Dale and Laidlaw (5) nor can any such activity be found in our own experiments.

Paton and Findlay (10) are strong advocates of guanidine or guanidine derivatives as the basis for parathyroid tetany. They point to the marked effect of a meat diet in bringing about parathyroid tetany and to Koch's (8) observation that methyl guanidine appears in the urine of parathyroidectomized animals. A full account of the history of the work of methyl guanidine and other compounds is found in their (10) work. They have shown physiologically, i.e., by tracings of tremors and the reaction of injected animals to electrical stimulation, that guanidine causes neuro-muscular phenomena similar to that following parathyroidectomy. They claim that the reactions after guanidine injection are identical with those of parathyroid tetany. In this contention they are supported by Farner and Klinger (7).

More recently Dragstedt et al (6) have shown that methyl guanidine is but one of the substances which under certain conditions may produce tetany symptoms. They have shown that parathyroidectomized animals are particularly susceptible to any kind of poisoning, and have produced what they term typical tetany seizures by stomach injections of yellow phosphorus. They have noted occurrence of tetany after bowel obstruction and have also obtained symptoms by the injection of intestinal content. In every case parathyroidectomized animals were more easily affected by such injections than were normal animals.

While it was not felt necessary to repeat the work of the investigators in this field, particularly the extensive work of Paton and Findlay and their co-workers on guanidine injections yet it seemed logical that some simple tests should be carried out in connection with our work on parathyroid tetany in the cat. To this end the experiments were planned simply as a comparison with the numerous cases of tetany which we have been able to observe. While no one who has studied the syndrome which may occur after parathyroid extirpation will lay down a single set of symptoms as typical of tetany yet the picture is fairly clear when large numbers of animals are under observation. The present writers, having become familiar with the tetany syndrome through operations upon nearly one hundred cats and dogs (11), (12) considered their experience sufficient to enable them to undertake an experiment such as presented here.

The experiments involved the use of the following substances: Methyl guanidine sulphate, methyl guanidine nitrate, trimethylamine, phosphorus, ergot, ammonia, intestinal content, creatine, creatinine and strychnine. The last named was added to the list purely for the sake of comparison with the other substances. The experiments are grouped

under three heads depending upon the method of administration: I, subcutaneous; II, intravenous injections; and III, oral administration.

I. Subcutaneous injections: The majority of the experiments were carried out by this method, as it seemed probable that the injected materials would be absorbed more slowly and uniformly than by other methods. It appeared probable, moreover, that the end result would be the same in any case if the substance used would produce symptoms comparable with parathyroid tetany.

TABLE 1

*Methyl guanidine sulphate**

CAT	WEIGHT	AMOUNT SUBCUTANEOUSLY PER KILO	TOTAL AMOUNT	RESULTS
55	2065	0.3	0.61	Tremors, spasms and death
56	4181	0.25	1.2	Tremors, prostration, death
57	3915	0.5	1.9	No epileptiform attacks, tremors, death
58	2975	0.2	0.59	Convulsions, prostration, death
N1	3000	0.33	1.00	Epileptiform attacks, fits, tremors, opisthotonus and death

* Methyl guanidine sulphate and nitrate and trimethylamine were obtained from the chemical research division of the Eastman Kodak Co. Creatine and creatinine were supplied by Dr. W. S. McEllroy of the Department of Biochemistry, University of Pittsburgh. Cats 55, 56, 57 and 58 had previously been thyroparathyroidectomized but had failed to develop tetany probably owing to the presence of accessory parathyroids.

TABLE 2

Methyl guanidine nitrate

CAT	WEIGHT	AMOUNT SUBCUTANEOUSLY PER KILO	TOTAL AMOUNT GRAMS	RESULTS
N2	3600	0.3	1.0	Convulsions, fits, tremors, prostration and death
N3	2600	0.1	0.26	Depressed, slight tremors, dead—24 hours
N4	2760	0.15	0.414	Tremors, fits and death, 24 hours
N5	2232	0.18	0.41	Depressed, no tremors, death—2 days
N6	1642	0.2	0.33	Tremors, twitchings and death—5 hours

Normal cats were used throughout the work except in one experiment where parathyroidectomized cats were employed. The animals had been kept in the laboratory, for the major part of the time unconfined, and were thoroughly accustomed to their environmental conditions before experimentation was begun. They were fed on a scrap diet consisting chiefly of cooked meat.

The animals were injected subcutaneously in the mid-dorsal region. A few intramuscular injections were given but subcutaneous injection is to be preferred.

a. Methyl guanidine experiments. Subcutaneous injections. Methyl guanidine sulphate when given subcutaneously causes death even with a relatively low dosage. In no case was the life of the animal prolonged, as is usually the case in tetany. The onset of toxic symptoms was sudden, violent in many cases, and lacked many of the features which result when the parathyroids are removed. There has been but one case in both this and the subsequent series in which typical "paw shaking" occurred. In most cases there is a tenseness or rigidity of the body, which is lacking in the fine tremor stage of tetany. This drug acts through the efferent arc according to Paton and Findlay. This same condition occurs also after parathyroid removal but there is such a contrast in the symptomatology of tetany and of guanidine poisoning that the difference can easily be observed.

The same general results hold for this series as noted for that receiving the methyl guanidine sulphate. The typical picture of a convulsion phase, tremor with the intervening phases of tetany, is lacking after injection. It will be noted that the end result of the two series is the same, although the sequence is slightly prolonged in this second series. This is concomitant with lower dosage. In no case giving the effect of tremor or convulsion was there any indication of these effects after the longest time of the experiments above recorded.

The symptoms following injections of methyl guanidine nitrate are easily distinguished from those following parathyroid removal in cats.

Trimethylamine gave no effect on subcutaneous injection outside of a purely local reaction and a general depression of the animal. There was no effect comparable to parathyroid tetany, although the dosage in both instances was quite high. This substance, according to Koch, is found in the urine of parathyroidectomized animals in unusual amounts, as compared with normals.

This substance is known to produce liver lesions and Dragstedt, et al have stated that symptoms similar to parathyroid tetany could be produced by the administration of the substance to parathyroidectomized dogs. In our normal cases, not even a generalized condition of depression could be obtained, though we employed higher dosage than did Dragstedt. The only effect was the sloughing of the skin over the area of injection.

The results with ergot were uniformly negative. There was no depression and no tremors on subcutaneous injection. The animals remained normal throughout, except for an extensive skin slough, which followed about five days after the injection. The drug was tested physiologically and was found to show the usual reaction to smooth muscle. Massive doses

TABLE 3

Trimethylamine

CAT	WEIGHT	AMOUNT PER KILO	TOTAL GRAMS	RESULTS
N27	2100	0.3	0.6	No effect could be noted
N28	3460	0.6	2.00	Depression, sleeps continually, no tremors, no fits

TABLE 4

Phosphorus

CAT	WEIGHT	AMOUNT PER KILO	TOTAL GRAMS	RESULTS
N9	3240	0.3	1.0	No effect except for the slough of a patch of skin at the area of injection
N10	3760	0.5	2.0	No effect, as above
N11	3016	1.2	4.0	No effect
N12	2769	1.8	5.0	No effect

TABLE 5

Ergot

CAT	WEIGHT	AMOUNT PER KILO	TOTAL	RESULTS
			grams	
N13	2700	0.37	1	No effect
N14	3240	0.60	2	No effect
N15	2279	1.7	4	No effect
N16	1497	3.3	5	No effect
N17	2672	1.4	4 (oral)	No effect
N18	2460	2.4	6 (oral)	No effect
Skin slough on subcutaneous and intramuscular injections				

TABLE 6

Ammonia

CAT	WEIGHT	AMOUNT PER KILO	TOTAL	RESULTS
			grams	
N19	2623	0.38	1	No effect
N20	2130	0.90	2	No effect

were used in an effort to produce some sort of a definite effect but none could be obtained. It seems highly improbable that the active principle of ergot is involved in any way in the tetany syndrome.

Subcutaneous injections of ammonium carbonate gave uniformly negative results.

Upon the basis of Dragstedt's idea that parathyroid tetany is due to absorption of toxic substances from the alimentary canal, substances probably of the nature of amines, resulting from the action of putrefactive bacteria upon proteins, these substances should be present in the intestinal contents. Dragstedt has secured tetany by injecting such contents into parathyroidectomized animals.

Although the injections, which were used in this series of normal cats are of massive dosage, no effect was obtained. The animals from which the contents were taken had been fed fresh meat for a day preceding the beginning of the experiment. The intestinal content was secured and the injections carried out as tabulated.

The series was separated into two parts. The first three animals received their injections as soon as possible after the contents were removed from the intestine. In the second, groups of two animals (N24) were injected after the material had stood for two days; (N25) were injected after the contents had been allowed to stand for five days. If decomposition products are capable of causing the effect of parathyroid tetany, the last two injections should have been potent in its production. Indol and skatol were present in large amounts.

With the exception of depression and vomiting, there was no reaction. The vomiting was not of the cachectic sort sometimes occurring after thyroparathyroidectomy.

With this substance the characteristic strychnine effect was obtained, convulsions, rigidity and death. While it may seem irrelevant to use this drug in an experiment involving a comparison with tetany, it may be noted that in several cases of methyl guanidine injection, the picture presented was quite as atypical for tetany as the sequence of events presented by strychnine poisoning. We employed it merely for comparison with the other substances injected.

II. Intravenous injections. All injections were carried out on normal cats, the jugular being opened and a cannula inserted. The materials were injected slowly in dilute solution. Two cases of methyl guanidine injection died before recovery from ether, due to too massive a dosage.

The three remaining cases of methyl guanidine injection were different in detail from each other and also were distinguishably different from parathyroid tetany. Upon recovery from the anesthetisation, one animal displayed marked tremors of the extremities. There were no tremors in the flank musculature nor were there convulsive movements of any sort. The animal was prostrate for six hours after injection after which recovery was normal. In the other two cases, the symptoms ran about the same course of events. Prostration was of longer duration in one animal in which a marked depression was noted. No tremors or convulsive move-

TABLE 7

Intestinal contents

CAT	WEIGHT	TOTAL AMOUNT	RESULT
		cc.	
N21	2941	5	No effect
N22	3617	10	Depressed, vomited, no tremors
N23	1920	5	Depressed, sick, no tremors, recovered
N24	1614	5	Very sick, vomited violently, depressed, recovered
N25	3290	5	Very sick, vomited, depressed, recovered

Injected with colon residue in which putrefaction was complete

TABLE 8

Creatine

CAT	WEIGHT	AMOUNT PER KILO	TOTAL	RESULTS
			grams	
29	2732	0.36	1	No effect
30	3340	0.90	3	No effect

TABLE 9

Creatinine

CAT	WEIGHT	AMOUNT PER KILO	TOTAL	RESULTS
			grams	
31	3760	0.40	1.5	No effect
32	3120	0.93	3.0	No effect
33	1691	1.7	3.0	No effect

TABLE 10

Strychnine

CAT	WEIGHT	AMOUNT PER KILO	TOTAL	RESULTS
			cc.	
60	1150	9.9	10	Dead five minutes
N7	1956	2.6	5	Strychnine convulsions—death
N8	2642	1.1	3	Convulsions—death

Death characterized by rigidity, which has a sudden onset after a few tremors

ments were noted in this animal in a period of recovery which lasted eight days. The remaining animal showed a marked depression with complete absence of tremor or convulsive attack.

Ergot was inert in all injections and no effect could be elicited.

Trimethylamine and ammonia both give a characteristic reaction, which

is different from parathyroid tetany. Both substances bring about coarse, jerky tremors of the extremities and body musculature while the animal is under anesthesia. This reaction occurred in both light and deep anesthesia. An overdosage or too rapid administration of either of these drugs will bring about death. The coarse jerky tremors persisted for some time after cessation of respiration and heart beat and give way to a ridigity similar to that obtained after strychnine. The tremors of parathyroid tetany disappear under deep anesthesia.

TABLE 11

Intravenous injections

SUBSTANCE	NUMBER OF CATS	DOSAGE PER KILO GRAMS	RESULTS
			Death in high dosage No tetany symptoms
Methyl guanidine sulphate	3	0.4 to 0.1	Depression in lower dosages
Methyl guanidine nitrate..	2	0.6 and 0.1	No tremors or induced tetany
Trimethylamine.....	4	0.1 and 0.02	Death in high dosage characterized by rigidity
Ammonia.....	2	0.6 and 0.2	Coarse, violent, jerky tremors persisting under anesthesia
Ergot.....	3	2.0 and 0.5	No effect

TABLE 12

Oral administration

SUBSTANCE	NUMBER OF CATS	DOSAGE PER KILO GRAMS	RESULTS
Methyl guanidine sulphate	2	2.0 and 1.0	Depression without tremor or convulsion. Uniform recovery
*Methyl guanidine nitrate.	2	0.8 and 0.5	
Trimethylamine.....	4	0.8 and 0.02	Nausea and depression, no tremors, no convulsions
Ammonia.....	3	0.7 and 0.01	
Ergot.....	6	4.0 and 0.7	No effect
Phosphorus.....	3	3.0 and 0.5	No effect

* Eight additional animals were given doses up to 2 grams per kilo without fatal results.

III. Oral administration. Administration of the drugs was accomplished by means of a small stomach tube introduced under anesthesia. This method was resorted to when the impossibility of administration by other methods had been demonstrated. The animals were closely observed for long periods after administration to be sure that none of the injected materials were lost by regurgitation.

The methyl guanidine series gave negative results in all of our cases. Dosage which would cause almost immediate reaction by subcutaneous injection induced merely a depression from which the animal recovered comparatively quickly. Trimethylamine and ammonium hydrate give a reaction of decided depression and nausea but no tremors or convulsive attacks.

Ergot and phosphorus were negative although given in massive dosage (cf. table).

Discussion. Our attempts to produce symptoms comparable to those of parathyroid tetany by means of injection or the administration of various substances to normal animals have failed in every case. Methyl guanidine sulphate and nitrate injected subcutaneously cause symptoms which tend to simulate those induced by parathyroid removal but the symptomatology and sequence present somewhat different pictures. There are individual effects produced by the guanidine series, which strongly simulates the response following parathyroid extirpation in the cat but the aggregate of symptoms in the two cases is hardly comparable.

It is to be emphasized that our work was done upon normal animals. It is possible that animals which have undergone a parathyroidectomy and which exhibit signs of parathyroid insufficiency may be acted upon more readily by these, and other substances. The evidence seems to point, in nearly all biological groups, to a lowering of resistance after parathyroidectomy not only to specific poisons but also to infection.

The inert condition of ergot and phosphorus in all series of experiments is constant. There was no effect of any sort observed which in any way could be compared with the symptoms of parathyroid tetany.

Trimethylamine and ammonia give perfectly definite results. Their effects are easily differentiated from those of the other substance and also from the effects after parathyroid extirpation. The coarse jerky tremors of the extremities, the tenseness and rigidity with lack of tremor in the body musculature together with the continuance of these reactions in deep anesthesia and even after cessation of heart beat and respiration are so different from parathyroid tetany that there is no possibility of confusion.

The method of administration plays an important part in the sequelae obtained. Methyl guanidine has practically no effect when administered by stomach tube; it is in the subcutaneous series that its action most nearly simulates tetany.

Trimethylamine and ammonia are most effective when administered intravenously. Their action, however, is not shown by other methods of administration.

Creatine and creatinine although related to the guanidine series failed to give any reaction with subcutaneous injection. Unfortunately, the amount of these substances available at the time of experiment was limited so that their effect in other series could not be noted.

Intestinal contents are non effective in subcutaneous injection. Stomach tube administrations were unsuccessful as the majority of the material was lost during recovery from anesthesia. The retained portion was sufficient to give a marked depression but there was no other effect noted.

While the reactions of cats to methyl guanidine injections and the reactions obtained after parathyroidectomy may have some effects in common, the results are essentially different in so many phases, that methyl guanidine intoxication can hardly be regarded as a primary causative factor in the production of parathyroid tetany.

SUMMARY

The reactions of normal cats to subcutaneous and intravenous injections and oral administration of certain substances, viz., methyl guanidine sulphate, methyl guanidine nitrate, trimethylamine, ergot, phosphorus, intestinal content, ammonia, creatine, creatinine and strychnine have been observed.

The symptoms occurring after injection of these substances were hardly comparable to the reactions of an animal suffering from parathyroid tetany.

The substances vary in effect according to their method of administration, methyl guanidine showing most marked reactions after subcutaneous injection and practically no effect after oral administration. Trimethylamine was active only when injected intravenously.

Ergot, phosphorus, creatine and creatinine were uniformly inert in all series.

Four thyroparathyroidectomized cats which had failed to show tetany symptoms due probably to the presence of accessory parathyroids, did not show tetany symptoms when injected subcutaneously with methyl guanidine sulphate.

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THE EFFECT OF THYROXIN AND ITS ACETYL DERIVATIVE ON AMPHIBIANS AND MAMMALS

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The primary object of the experiments recorded here was to test whether or not there exists a fundamental difference between mammals and amphibians in regard to their physiologic responses to thyroid and iodine administration. Certain experiments of Kendall (1) and Swingle (2), (3), (4), (5) indicated that differences existed between these two vertebrate groups with respect to the chemical grouping in the molecule responsible for the changes induced in each, following thyroid administration. For instance, iodotyrosin, iodoserumalbumin, iodoserumglobulin and iodocasein, when fed to thyroidless or pituitaryless amphibian larvae, bring about a rapid and precocious metamorphosis, the effect produced upon these organisms being similar to those induced by thyroid feeding except that the metamorphic changes occur somewhat less rapidly. The same substances exert no influence upon amphibian transformation without the iodine in the molecule, nor can other halogens such as bromine be substituted for the iodine and the substances still retain their activity. However, it is pretty well established that iodized proteins and amino acid such as iodotyrosin, iodocasein, iodoserumalbumin and globulin exhibit no demonstrable effects when fed to mammals suffering from cretinism or myxedema and do not, when tested on normal animals, induce those physiologic responses which usually follow thyroid administration in this group.

Oswald (6) has studied the physiological properties of several iodized proteins and compared them with thyroid. He found, for example, that iodocasein, iodotyrosin and iodoserumalbumin are inactive when administered to mammals and do not exert thyroid-like effects.

Hellin (7) reported years ago that iodized albumin and nucleo-albumin prepared from the spleen are inactive when given to mammals, and Blum (8) in the year following publication of Hellen's paper reported that iodized albumin has practically no effects upon metabolism.

Strouse and Voegtlin (9) failed to observe any thyroid-like effects on the nitrogen metabolism or on the blood pressure of normal dogs, nor

was there any favorable effect on the condition of myxedematous and cretinous mammals following administration of diiodotyrosin.

The following experiment of Kendall (10) is an excellent illustration of the point involved, namely, that the substances inducing thyroid-like effects, i.e., metamorphic changes in amphibia have no influence upon the metabolic rate of mammals, and that the two classes of vertebrates probably react to quite different chemical groups in the thyroid hormone.

Kendall observed that injections of pure thyroxin into mammals are followed by a very definite and marked physiologic response. But when the hydrogen of the imino group in the thyroxin is replaced by acetyl, the substance loses its physiologic activity and there follows no demonstrable effect upon the metabolic rate. This emphasizes the importance of the imino group in thyroxin (insofar as the metabolic effect upon mammals is concerned) and minimizes the importance of iodine in the molecule. Bearing in mind the effects of certain organic iodine compounds upon tadpole metamorphosis, Kendall tested the acetyl derivative of thyroxin on tadpoles, for if the metamorphosis depends only upon the increase in the basal metabolic rate of the larvae then thyroxin should increase the rate of metamorphosis, but the acetyl derivative involving the imino group should not. If, however, iodine alone is concerned in accelerating metamorphosis, then both thyroxin and the derivative should affect the transformation. It was found that both thyroxin and the acetyl derivative would induce a rapid metamorphosis of the bullfrog tadpole. The conclusion was that thyroxin appears to have two separate and distinct functions: the effect upon the mammalian metabolic rate which is brought about by the CO-NH groups within the molecule; and the physiological changes involved in the metamorphosis of the tadpole due to iodine contained in the molecule.

Kendall's experiment is of great interest to workers on amphibian metamorphosis but, so far as we are aware, has never been published except in an abstract of about two hundred and fifty words, and all details are lacking. We have repeated the experiment on an extensive scale using normal men for mammalian material, and thyroidectomized and hypophysectomized tadpoles for the metamorphosis phase of the problem. In the course of the work interesting data relating to the physiological activity of thyroxin when taken orally were obtained.

We take this opportunity of expressing our obligation to Dr. E. C. Kendall of the Mayo Clinic for acetylating the thyroxin.

Experimental. *A. Amphibians.* Several hundred Rana sylvatica embryos were thyroidectomized and hypophysectomized, and kept until several months after the period of metamorphosis normal for this species had elapsed, and until all of the normal unoperated control animals from the same egg clusters had transformed into frogs. It will be recalled that

thyroidless and pituitaryless frog tadpoles do not metamorphose but permanently retain their larval characters. Sixty operated animals, thirty thyroidless and thirty pituitaryless, were injected intraperitoneally with small quantities of acetyl thyroxin, and a like number injected with an equal amount of pure thyroxin. An effort was made to study quantitatively the effects of thyroxin and its acetyl derivative when administered to tadpoles, but unfortunately small amounts of the injected solutions were in most cases lost following withdrawal of the needle from the peritoneal cavity or subcutaneous lymph sinuses. This was true despite the fact the animals were first anesthetized in chloretone solution. Owing to the loss of minute quantities of the injected substances, efforts to obtain exact quantitative data on the physiologic activity of the thyroxin and acetyl thyroxin were abandoned. Qualtitatively, however, the two substances behaved alike; both thyroidless and pituitaryless larvae responded to the injection by very rapid and precocious metamorphosis, the larger the amount given, the more rapid the transformation. We were able to induce marked metamorphic changes, such as development of the fore and hind limbs, tail resorption and development of the typical frog mouth, in thyroidless and hypophysectomized tadpoles, within nine days from the date of first administering the thyroxin and its acetyl derivative. Merely feeding a small amount of crystals of both substances to tadpoles was sufficient to induce very marked metamorphic responses. Several animals had well-developed fore legs (generally only the left one through the operculum) within five days of the date of first feeding. The rate of metamorphic change induced was so rapid that the animals invariably died before tail resorption was completed and in many cases before both fore legs appeared. The reaction was comparable in every way to that following the administration of thyroid extract or tissue.

Our experiments on administration of thyroxin and its acetyl derivative to thyroidectomized and hypophysectomized tadpoles confirm the observations of Kendall (10) who performed a similar experiment on normal bullfrog larvae, possessing intact thyroid and pituitary glands. It is probable, though our experiments do not prove it, that quantitatively thyroxin is somewhat more potent in inducing the metamorphic response of tadpoles than is the acetyl derivative. In a personal communication to one of the writers (W. W. S.), Kendall stated that his experiments showed that pure thyroxin is somewhat more active than the acetyl derivative in bringing about metamorphic change in tadpoles. However, there can be no question about the potency of acetyl thyroxin as an accelerator of anuran transformation for it is a highly active metamorphosis-inducing agent. Owing to lack of sufficient material we have not tested acetyl thyroxin on urodele larvae.

The second step in the work was to test the physiological activity of thyroxin and its acetyl derivative upon mammals. Both of the substances had proven to be active on amphibians but according to theory only thyroxin should show physiological activity when administered to mammals.

B. Mammals. Four adult, healthy, male individuals were used in the experiments, consisting of the writers and Mr. K. Donahue, Technical

TABLE 1

Record of pulse and general condition of a 33-year old male individual following an intravenous injection of 10 mgm. of thyroxin

DATE 1924	PULSE RATE			REMARKS
	4–6 p.m., highest	7:00 a.m., lowest	Average	
March 27	80	72	78	
March 28	80	70	78	
March 29	82	66	79	
March 30	84	72	80	
March 31	80	70	79	
April 1	84	70	80	Weight, 142 lbs. 10 mgm. thyroxin taken intravenously
April 2	112	84	104	Violent headache, dizziness, diarrhea
April 3	104	94	100	Headache, dizziness, muscular weakness
April 4	110	101	104	Symptoms same as before. Shortness of breath following slight exertion. Dizziness
April 5	108	100	104	Headache disappeared. Muscular weakness marked. Feel ill
April 6	104	95	100	Headache again. Dizziness. Muscular weakness
April 7	115	90	100	Headache and dizziness disappeared. Muscular weakness marked
April 8	109	92	97	Symptoms abating. Feel better
April 9	100	88	94	Weight, 140 lbs. Symptoms disappeared
April 10	102	88	94	
April 11	94	80	90	
April 12	90	80	87	Weight, 141.5 lbs.
April 13	88	77	85	
April 14	89	78	84	

Assistant in the Osborn Zoölogical Laboratory. A fifth man was employed in part of the work involving oral administration of thyroxin but after taking 54 mgm. he contracted severe tonsilitis and was not utilized further in the experiments.

We were not in a position to measure basal metabolism or in fact to make any kind of metabolic tests, and throughout were forced to rely entirely upon the pulse rate, nervous symptoms and variations in body weight indicating physiological response upon the part of the organism

to the administered thyroxin. This is a legitimate procedure and has been employed by Carlson (11) and Kendall (1) in testing the response of the individual to thyroid products.

According to Kendall (1) thyroxin, even in small amounts, will produce toxic effects. The severity of the toxic symptoms following administration of Alpha iodine (thyroxin) to normal individuals varies greatly, but in general is as follows: If to a normal individual with a pulse-rate of about 75 and a weight of about 75 kilos, 3 mgm. of thyroxin is given per

TABLE 2

Record of pulse and general condition of a 21-year old male individual following an intravenous injection of 10 mgm. of thyroxin

DATE 1924	PULSE RATE			REMARKS
	4–6 p.m., highest	7:00 a.m., lowest	Average	
March 28	85	59	70	
March 29	79	61	68	
March 30	82	61	70	
March 31	80	56	69	
April 1	84	59	71	Weight, 175 lbs. Took 10 mgm. thyroxin intravenously
April 2	98	74	86	
April 3	120	83	105	Muscular weakness. Dizziness, headache and diarrhea
April 4	94	89	92	General weakness and headache
April 5	86	74	82	Headache and dizziness
April 6	92	78	88	Muscular weakness
April 7	125	78	100	
April 8	121	83	100	Ill in bed
April 9	100	88	96	Muscular weakness. Out of breath following slight exertion
April 10	105	80	97	
April 11	99	81	95	Symptoms disappeared
April 12	101	78	90	Weight, 174 lbs.

day, on the day following the first dose there is no apparent change except a slightly increased pulse rate. If the same daily dose is continued the patient slowly reacts and in the course of eight or ten days, nervous symptoms develop, the pulse rate is around 130 to 140, there is a tendency to perspire more freely on exertion, and the individual may be short of breath and easily tired. At first there is increased appetite, but later loss of appetite, nausea and sometimes diarrhea develops. During this time a loss of weight varying from 1 to 5 kilos may occur, though this is not invariable. If smaller doses are given, the severity and course of symptoms are modified, but the patient will react to very small amounts of thyroxin. If larger doses than 3 mgm. are given, there results a serious condition

in which the above symptoms are exaggerated. Kendall states that some individuals develop severe symptoms following administration of as little as $\frac{1}{75}$ to $\frac{1}{25}$ of 1 mgm. thyroxin per body weight per day.

The thyroxin and its acetyl derivative were given orally and intravenously and to our surprise the thyroxin proved active only when given by vein. Tables 1 and 2 are records of the pulse rate and general condi-

TABLE 3

Record of pulse and general condition of a 33-year old male individual following an intravenous injection of 10 mgm. of acetyl thyroxin

DATE 1924	PULSE RATE			REMARKS
	4–6 p.m., highest	7:00 a.m., lowest	Average	
May 6	72	74	76	
May 7	79	66	77	
May 8	80	65	78	
May 9	81	68	77	
May 10	80	70	78	
May 11	78	67	76	
May 12	80	70	78	
May 13	80	70	78	10 mgm. of acetyl thyroxin taken intravenously at 4 p.m., weight, 142 lbs.
May 14	80	66	77	No symptoms
May 15	80	70	78	No symptoms
May 16	77	68	76	No symptoms
May 17	80	70	78	No symptoms
May 18	80	69	75	No symptoms
May 19	81	66	79	No symptoms
May 20	78	70	77	No symptoms
May 21	80	66	76	Weight, 141¾ lbs.
May 22	80	65	77	No sympotms
May 23	78	67	75	No symptoms
May 24	80	70	77	No symptoms
May 25	79	68	75	No symptoms
May 26	80	66	78	Weight, 141.5 lbs.

tion of two individuals following an intravenous injection of 10 mgm. of thyroxin.

Following the injection there was a lag of approximately twenty-four hours before the first symptoms made their appearance. The rise in pulse rate recorded in the table and the statement of the principal symptoms does not adequately express the feelings of the two injected individuals. It is interesting to compare our tables 1 and 2 with the table in Carlson's (11) paper which details the results of continuous thyroid feeding to a normal, adult man. The symptoms induced by thyroid feeding and by intravenous injection of thyroxin are identical. Tachy-

cardia did not occur. Our experiment indicates that normal individuals react more violently to injections of thyroxin than do myxedematous or cretinous patients. So far as we are aware, 10 mgm. of thyroxin when given intravenously to such patients does not evoke toxic symptoms or at any rate to the extent observed by us. In the normal individual the thyroxin injected is superfluous and over and above that suited to the needs of the organism and induces untoward symptoms. Myxedematous

TABLE 4

Record of pulse and general condition of a 21-year old male individual following an intravenous injection of 10 mgm. of acetyl thyroxin

DATE 1924	PULSE RATE			REMARKS
	4–6 p.m., highest	7:00 a.m., lowest	Average	
May 6	79	59	73	
May 7	75	57	68	
May 8	78	60	72	
May 9	76	60	73	
May 10	77	56	70	
May 11	80	58	75	
May 12	76	56	74	
May 13	74	59	67	10 mgm. of acetyl thyroxin taken intravenously 4:15 p.m., weight, 173.5 lbs.
May 14	72	64	68	No symptoms
May 15	69	57	66	No symptoms
May 16	73	55	68	No symptoms
May 17	75	61	69	No symptoms
May 18	77	58	68	No symptoms
May 19	79	60	70	No symptoms
May 20	81	56	73	No symptoms
May 21	79	58	68	Weight, 174 lbs.
May 22	80	55	71	No symptoms
May 23	80	57	70	No symptoms
May 24	78	60	69	No symptoms
May 25	81	61	72	No symptoms
May 26	79	59	68	Weight, 174 lbs.

or cretinous individuals, on the contrary, demand large amounts of thyroid hormone and toxic effects are not produced following its administration in amounts such as used by us.

Intravenous injections of acetyl thyroxin into the same two individuals receiving thyroxin proved negative. The injections were given several weeks (approximately four) after the last thyroxin was taken. Tables 3 and 4 show clearly that acetyl thyroxin is physiologically inert when injected into mammals.

For oral administration the 2-mgm. tablets of the sodium salt of thyroxin, as prepared by E. R. Squibb & Sons under license from the University of Minnesota, were used. The acetyl derivative of thyroxin was administered orally in gelatine capsules. The tablets and capsules were taken about one hour before meal time and the pulse was taken five

TABLE 5

Record of pulse and general condition of 33-year old male individual fed acetyl thyroxin

DATE 1923	ACETYL-THYROXIN TAKEN	PULSE RATE			REMARKS
		4:00–6:00 p.m., highest	7:00 a.m., lowest	Average	
	mgm.				
November 23	None			80	Weight, 141 lbs.
November 24	None			81	
November 25	None			82	Total amount acetyl thyroxin taken 28 mgm.
November 26	None			79	
November 27	2	80	75	80	
November 28	2	82	76	80	
November 29	2	80	74	77	
November 30	2	82	76	79	
December 1	2	80	74	78	
December 2	2	78	68	73	
December 3	2	78	68	74	
December 4	2	80	68	74	
December 5	2	80	75	77	
December 6	2	80	73	72	
December 7	2	80	68	75	
December 8	2	78	68	72	
December 9	2	80	69	74	
December 10	None	78	73	74	
December 11	2	75	72	73	
December 12	None	80	70	74	
December 13	None	78	68	72	
December 14	None	80	69	76	
December 15	None	78	64	78	
December 16	None	80	65	78	
December 17	None	80	68	78	Weight, 141 lbs.

or six times a day beginning in the morning just before arising. Only the highest, lowest and average pulse are given for each day. After discontinuing the thyroxin the pulse was taken each day for a week in order to see if any rise occurred but these data are not included in the tables because in all cases the results were negative.

A survey of tables 5, 6, 7 and 8 shows quite clearly that acetyl thyroxin in doses of 2 mgm. per day for about two weeks has little if any effect upon the pulse rate. It was then decided that probably 2 mgm. per day

are insufficient to induce a physiological response. The reason this quantity was chosen was because the manufacturers of thyroxin state: "Concerning the clinical use of thyroxin, the following facts have been determined: a normal individual cannot take 2 milligrams of thyroxin a day continuously without developing symptoms of hyperthyroidism."

The acetyl thyroxin feeding was discontinued December 13. On January 10, one individual was fed 2 mgm. of pure thyroxin per day

TABLE 6

Record of pulse and general condition of 26-year old male individual fed acetyl thyroxin

DATE 1923	ACETYL-THYROXIN TAKEN	PULSE RATE			REMARKS
		Highest	Lowest	Average	
	mgm.				
November 23	None			84	Total amount acetyl thyroxin 28 mgm.
November 24	None			84	
November 25	None			76	
November 26	None	82	74	78	
November 27	2	82	76	80	
November 28	2	88	76	82	
November 29	2	88	74	86	
November 30	2	86	80	82	
December 1	2	90	80	85	
December 2	2	84	82	83	
December 3	2	86	84	84	
December 4	2	82	80	81	
December 5	2	84	80	82	
December 6	2	82	80	81	
December 7	2	84	80	82	
December 8	2	82	80	81	
December 9	2	84	80	82	
December 10	None	84	80	82	
December 11	2	82	80	81	
December 12	None	82	80	81	
December 13	None	84	80	82	
December 14	None	82	80	82	
December 15	None	82	80	81	
December 16	None	82	80	81	

for twelve days and then fed acetyl thyroxin in strong doses until a total of 76 mgm. of the acetyl derivative had been taken. The results are summarized in table 8 and show clearly that 24 mgm. of thyroxin and 76 mgm. of the acetyl derivative taken in strong doses continually (except for three days) for twenty-six days exerts no influence upon the pulse rate.

Our negative findings with acetyl thyroxin on man are in agreement with those of Kendall who used this substance on dogs, and with our results on intravenous injection. We had anticipated negative results following administration of the acetyl thyroxin but the consistently negative findings in the case of the orally administered thyroxin was a surprise.

TABLE 7

Record of pulse and general condition of 21-year old male individual fed acetyl thyroxin

DATE 1923	ACETYL-THYROXIN TAKEN	PULSE RATE			REMARKS
		Highest	Lowest	Average	
	mgm.				
November 23	None			72	Weight, 170 lbs.
November 24	None			72	
November 25	None			72	Total amount of acetyl thyroxin taken, 30 mgm.
November 26	None			73	
November 27	2	78	64	73	
November 28	2	78	66	72	
November 29	2	78	65	74	
November 30	2	70	68	70	
December 1	2	74	60	66	
December 2	2	74	62	70	
December 3	2	72	60	72	
December 4	2	72	60	69	
December 5	2	72	60	69	
December 6	2	70	59	66	
December 7	2	67	56	66	
December 8	2	80	70	72	
December 9	None		65	70	
December 10	None	72	62	67	
December 11	2	72	58	68	
December 12	2	72	64	69	
December 13	2	76	72	74	
December 14	None	73	70	71	
December 15	None	72	68	70	
December 16	None	70	66	68	
December 17	None	71	68	70	
December 18	None	72	66	69	Weight, 170 lbs.

The data on thyroxin feeding given in tables 9, 10 and 11 clearly indicate that this substance when given orally in the quantity used by us exerts little effect upon the pulse rate of normal, healthy individuals. Table 11 is of particular interest because the individual represented had never received any acetyl thyroxin or thyroxin previous to his first dose on February 15, whereas the other three individuals had previously (about

TABLE 8

Record of pulse and general condition of 21-year old male individual fed sodium salt of thyroxin and acetyl thyroxin

DATE 1924	THYROXIN TAKEN	PULSE RATE			REMARKS
		Highest	Lowest	Average	
	mgm.				
January 8	None			66	Total amount of thyroxin taken, 24 mgm. Acetyl thyroxin, 76 mgm.
January 9	None			65	
January 10	2	72	66	69	
January 11	2	70	65	68	
January 12	2	72	68	70	Weight, 170 lbs.
January 13	2	80	70	76	
January 14	2	78	72	75	
January 15	2	75	66	71	
January 16	2	120	69	88	High pulse taken after brisk walk
January 17	2	75	70	72	
January 18	2	112	68	83	High pulse taken after brisk walk
January 19	2	80	71	76	
January 20	2	76	62	71	
January 21	2	81	64	73	
	ACETYL-THYROXIN				
January 22	4	75	69	71	
January 23	4	68	60	65	
January 24	4	70	66	68	
January 25	4	70	60	65	
January 26	4	78	68	72	
January 27	4	75	60	68	
January 28	6	74	62	66	
January 29	6	72	58	67	
January 30	6	80	60	71	
January 31	6	78	62	70	
February 1	None	72	64	68	
February 2	None	75	66	71	
February 3	None	77	65	70	
February 4	8	80	67	71	
February 5	8	80	67	74	
February 6	8	79	68	73	
February 7	4	80	60	69	
February 8	None			70	Weight, 174 lbs.
February 9	None			71	

one month) taken 28 mgm. of acetyl thyroxin. The data in table 11 rule out the possibility of any tolerance for thyroxin having developed, thus

TABLE 9

Record of pulse and general condition of 33-year old male individual fed sodium salt thyroxin

DATE 1924	THYROXIN TAKEN	PULSE RATE			REMARKS
		2:00 p.m., highest	7:00 a.m., lowest	Average	
	mgm.				
January 8	None	74	74	74	Total amount of thyroxin taken, 112 mgm.
January 9	None	76	65	70	
January 10	2	78	72	75	
January 11	2	80	72	78	
January 12	2	75	69	74	Weight, 138.75 lbs. on this date
January 13	2	78	73	75	
January 14	2	90	72	80	
January 15	2	83	70	75	
January 16	2	83	72	88	
January 17	2	81	72	72	
January 18	2	82	71	83	
January 19	2	81	75	76	
January 20	2	83	76	71	
January 21	2	112	74	73	112 at 2:00 p.m.
January 22	4	93	72	71	90 at 6:00 p.m.
January 23	4	83	72	65	74 at 10:00 p.m.
January 24	4	97	55	68	Pulse irregular
January 25	4	91	74	65	
January 26	4	94	72	72	
January 27	4	90	74	68	
January 28	6	102	65	80	Pulse down to 72 at 6:00 p.m.
January 29	6	100	70	80	
January 30	6	97	70	78	Pulse down to 72 at 6:00 p.m.
January 31	9	85	70	83	Crystalline thyroxin taken
February 1	None	97	63	78	
February 2	None	84	72	76	
February 3	None	87	73	78	
February 4	8	102	75	78	Thyroxin taken at 5:30 p.m.
February 5	8	90	74	78	
February 6	8	79	74	78	
February 7	5	83	72	78	Weight, 141 lbs. on this date
February 8	8	75	66	76	Crystalline thyroxin taken
February 9	None	78	72	76	

accounting for the more or less negative findings. None of the persons taking thyroxin by mouth reported any ill effects whatever. Dizziness, headache, nervousness or other symptoms were not noted.

Two thyroxin-fed individuals report a loss of weight of three and four pounds respectively, and one, a two and one-quarter pound gain. The

TABLE 10

Record of pulse and general condition of 26-year old male individual fed sodium salt of thyroxin

DATE 1924	THYROXIN TAKEN	PULSE RATE			REMARKS
		Highest	Lowest	Average	
	mgm.				
January 8	None	84	80	82	Total amount thyroxin taken, 106 mgm.
January 9	None	82	78	80	
January 10	2	84	80	82	
January 11	2	90	82	86	
January 12	2	80	78	79	Weight, 169 lbs.
January 13	2	80	76	79	
January 14	2	94	80	85	
January 15	2	102	80	87	80 at 11:00 a.m., 102 at 8:00 p.m., 80 at 11:00 p.m.
January 16	2	82	74	79	
January 17	2	80	78	79	
January 18	2	82	78	80	
January 19	2	80	70	76	
January 20	2	76	74	75	
January 21	2	91	90	91	
January 22	4	105	80	88	
January 23	4	84	74	80	
January 24	4	82	80	81	
January 25	4	84	80	82	
January 26	4	94	76	83	
January 27	4	80	72	77	
January 28	6	90	82	86	
January 29	6	90	86	89	
January 30	6	96	80	87	
January 31	8	90	70	81	
February 1	None	84	80	83	
February 2	None	82	80	81	
February 3	None	84	80	82	
February 4	8	91	84	87	
February 5	8	86	80	83	Crystalline thyroxin taken
February 6	8	82	80	81	
February 7	8	84	82	83	Weight, 165 lbs.
February 8	None	84	80	82	
February 9	None	82	80	81	
February 10	None	84	80	82	
February 11	None	90	82	86	

individual reporting the four-pound loss during an interval of twenty-five days states that his weight fluctuates considerably, and considers the weight loss of little significance. On the other hand, the individual

reporting the three-pound loss states that his weight remains fairly constant and varies little. The weight loss of this individual occurred during an interval of eighteen days. The third individual reporting a gain in weight states that his weight varies considerably. The fourth man receiving 38 mgm. of thyroxin and 76 mgm. of acetyl thyroxin gained four pounds and states that his weight is not subject to variation. It is unfortunate that daily weighings were not made. Kendall (1) remarks that patients receiving group A thyroid constituents (thyroxin) and presenting very marked physiological response such as greatly in-

TABLE 11

Record of pulse and general condition of 19-year old male individual fed sodium salt of thyroxin

DATE 1924	THYROXIN TAKEN	PULSE RATE			REMARKS
		6:00 p.m., highest	7:30 a.m., lowest	Average	
	mgm.				
February 15	8	76	71	72	Weight, 146 lbs.
February 16	8	80	59	80	Total amount thyroxin taken, 104 mgm.
February 17	8	76	70	75	
February 18	8	85	65	78	
February 19	8	78	50	63	
February 20	8	80	55	67	
February 21	8	81	59	75	
February 22	8	77	65	70	
February 23	8	80	65	72	
February 24	8	77	69	72	
February 25	8	78	66	72	
February 26	8	91			Only one pulse rate taken
February 27	8	71	69	70	
February 28	None	79	70	74	
February 29	None	70	68	69	
March 1	None			71	
March 2	None			73	
March 3	None			71	Weight, 143 lbs.
March 4	None			72	

creased pulse rate (150 per minute) and nervous symptoms, may or may not show loss of weight.

Discussion. It is generally stated that thyroxin represents the active principle of the thyroid hormone, and a considerable amount of clinical and experimental data, especially basal metabolism tests, support this contention. Clinical work on the treatment of cretinism and myxedema with intravenous injections of thyroxin shows clearly that these conditions of hypothyroidism can be successfully treated with this substance. Myxedematous patients at the Mayo Clinic are treated with thyroxin in a

quantitative manner. If the metabolic rate is 40 per cent below normal they are brought to normal by the administration of 20 mgm. of thyroxin. Plummer has shown that a quantitative relationship exists between thyroxin and the basal metabolic rate. The administration of 1 mgm. of thyroxin to an adult weighing approximately 150 pounds increases the basal metabolic rate 2 per cent. The administration of 2 mgm. increases it 4 per cent; the administration of 10 mgm., 20 per cent. The experimental work on mammals shows that thyroxin influences the metabolic rate of these forms qualitatively in the same fashion as thyroid extract or tissue.

We were greatly surprised to find that the sodium salt of thyroxin and the pure crystalline substance were practically inactive when taken orally by normal individuals. So far as we are aware, this fact has not been reported in the literature. Our experiments indicate that thyroxin should be administered to myxedematous and cretinous patients intravenously if thyroid-like effects are to be produced. It will be recalled that the physiologic activity of thyroid tissue or extract is not diminished by passage through the alimentary canal, thus indicating that thyroxin differs somewhat from the thyroid hormone as it actually exists within the gland.

Careful studies on the comparative physiologic activity of thyroid and thyroxin are few, but two being known to the writers. Utilizing the acetonitrile test, Hunt (12), in a very extensive series of experiments, attempted to determine if thyroxin when fed to mice has an action qualitatively and quantitatively like that of thyroid, and if thyroxin in proportion to its iodine content differs in the degree of its activity from thyroid. His tables show that thyroxin has the qualitative activity of thyroid; its quantitative activity also being vastly greater than that of any other iodine-containing substance except the thyroid. But in not a single instance among the numerous experiments was the thyroxin, in proportion to its iodine content, as active as any of the normal adult thyroids used. Hunt's protocols show that thyroid tissue, in proportion to its iodine content, is about one and one-half times as active as thyroxin, when the latter was given either orally or intravenously. It is interesting to note that the thyroxin when given orally to mice exerts a qualitative effect like thyroid in raising the resistance of these animals to acetonitrile. It seems that in mice the thyroxin is either not destroyed in the alimentary canal as is probably the case in man, or else the increase in the resistance of mice to acetonitrile after thyroid or thyroxin administration depends upon some other chemical grouping in the hormone than that primarily responsible for the unique effect of this substance upon mammalian metabolism.

Cameron and Carmichael (13) using as a criterion the retarding effects of thyroid administration upon the growth of rats, found thyroid to be from two to four times as active as thyroxin when fed in equi-iodine dosage. They suggested that probably the thyroxin was destroyed in the alimentary tract to a greater extent than was the thyroid. Hunt reports, however, that although the protective action of thyroxin to the lethal effects of acetonitrile developed more rapidly when the drug was given intravenously than when fed, the degree of protection ultimately attained was not materially greater. However, there is some additional indirect evidence that thyroxin when given orally is less active than when administered intravenously; for instance Snell, Ford and Rowntree (14) state "we have had some difficulties with administration of thyroxin by mouth, but we have succeeded in holding some of the patients (cretins) at a normal metabolism by oral administration." The authors had no trouble whatever when the drug was given intravenously. The graph in figure 3 of their paper is suggestive in this connection.

The fact that both thyroxin and its acetyl derivative are active in inducing amphibian transformation, whereas only the thyroxin shows physiologic activity when administered to mammals is excellent evidence that amphibians and mammals differ considerably in regard to the nature of the chemical groupings within the thyroid hormone to which they respond. The substitution of acetyl for the hydrogen of the imino of thyroxin completely destroys its physiologic activity insofar as the metabolic rate of mammals is concerned, but this substitution apparently has no influence upon the iodine-containing group in the molecule responsible for amphibian metamorphosis.

SUMMARY

1. The experiments were designed to test the view that the physiologic responses of amphibians and mammals to thyroid administration depend upon different chemical groupings within the hormone.

2. Both thyroxin and its acetyl derivative are highly active agents in inducing the metamorphosis of thyroidectomized and hypophysectomized tadpoles when either fed or injected.

3. Thyroxin when given intravenously to normal, adult men exerts a profound physiologic response, comparable to that evoked by excessive thyroid feeding.

4. Oral administration of thyroxin gave practically negative results despite the large dosage employed. Thyroxin is apparently destroyed within the alimentary canal of man.

5. Acetyl thyroxin when intravenously injected into normal individuals exerts no physiologic response. Oral administration of this substance likewise proved negative.

6. Chemically the only difference between thyroxin and the acetyl derivative is the substitution of acetyl for the hydrogen of the imino group.

7. Amphibian metamorphosis depends upon a peculiar organic iodine complex as shown by the effect of acetyl thyroxin, iodotyrosin, iodoserumalbumin, iodoserumglobulin and iodized casein upon these forms.

8. The effect of thyroid upon mammalian metabolism is apparently unique and is not induced by other organic iodine compounds which metamorphose amphibians. The metabolic responses of mammals and the metamorphic responses of amphibians to thyroid are hardly to be compared as they are apparently due to quite different chemical groupings within the hormone although the iodine seems essential for the activity of both chemical groups, i. e., the group responsible for mammalian metabolism and the group for amphibian metamorphosis.

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AN EXPERIMENTAL STUDY OF THE DEVELOPMENT OF THE MOUTH IN THE AMPHIBIAN EMBRYO

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SEVEN PLATES

CONTENTS

Introduction 311
Materials, methods, and normal development 314
Experimental 321
A. Regeneration experiments 322
B. Transplantation experiments 333
1. Transplantation of homoplastic gill ectoderm to stomodaeal region (StG series) 334
2. Transplantation of homoplastic ventral body wall ectoderm to stomodaeal region (StV series) 346
3. Transplantation of heteroplastic frog ectoderm to stomodaeal region 348
4. Transplantation of homoplastic stomodaeal ectoderm to gill region (GSt series) 350
Discussion 353
Summary 361
Literature cited 363

INTRODUCTION

It is difficult in adult vertebrates and in embryos after the breaking of the oral plate which separates stomodaeum and foregut to determine the exact limits of ectoderm and endoderm in the mouth cavity. As a result, the literature dealing with the development of the mouth includes more or less speculation as to the precise boundaries of the two germ layers and their derivatives. In spite of this uncertainty, the idea is prevalent that the presence of teeth, structures presumably homologous with the placoid scales of Elasmobranchs and to some extent the presence of taste buds in any

portion of the oropharyngeal cavity, is a proof that ectoderm has existed in this region. Typical instances of this opinion may be found in the works of Kingsley and Thyng ('04), Fahrenholz ('15), Tomes ('14), Johnston ('09, '10), Landacre ('21), and others, which deal largely with fishes and amphibia.

Occasionally, however, one finds the value of these criteria questioned, implying also doubt as to the strict specificity of the germ layers in the development of the structures of the mouth. In the case of the enamel organs of the teeth, this doubt appears in the writings of Goette ('75), Ryder ('86, quoted by Wortman), Prince ('92), Göppert ('06), Kerr ('02, '09, '10, '19), and more recently of Cook and Neal ('21), while Johnston and Cook and Neal challenge specificity from the standpoint of the development of the taste buds. Kerr, in fact, not only denies the necessity of an ectodermal contribution in the development of teeth and claims that endoderm may form enamel organs, but also disagrees with the conception of an ectodermal invagination in the development of the mouth of the Dipnoans (Lepidosiren and Protopterus) and the Urodeles (Amblystoma and Triton). "It is, as it were, as if an influence were spreading inwards from the external epiblasts, gradually transforming the original 'endoderm' yolk-laden cells into ectoderm like itself" or a "process of conversion in situ." Other investigators, however, Goette, Kingsley and Thyng, Greil ('08), Johnston and Landacre describe this invagination of ectoderm from which enamel organs of maxillary, mandibular, and vomerine teeth are said to be derived, and Kingsley and Thyng and Landacre, disagreeing with Johnston, interpret it as representing a modification in the formation of the stomodaeum due to the thickened endodermal component of the oral plate.

Further than this the study of the development of the urodele tooth still presents certain unsettled questions. It has been well established by Hertwig ('74), Wiedersheim ('90), and Röse ('94, '95) that the pulp of a tooth consists of a number of cells, and these pulp cells have been identified by Platt ('93, '94) in Necturus and by Landacre in Pletho-

don(?) as mesectoderm and not epithelium, as Leydig ('67) had asserted. This mesectoderm is derived from the trigeminal portion of the neural crest. The former group of workers also confirmed the investigation of Heincke ('73), who found both enamel and dentine in the composition of a tooth and corrected the statements of Gegenbaur[1] ('62), and S. Sirena ('71–'72), who thought each tooth the product of a single cell, although covered by an epithelial cap, and made up only of dentine secreted by this single odontoblast. The unestablished points concern the method of origin of the earliest tooth anlagen in the urodele and the part of the definitive papilla which instigates development. S. Sirena and later Wiedersheim and Röse had asserted that the earliest tooth papilla (splenial) 'pushed up freely' into the mucous membrane of the lower jaw after the fashion of a placoid scale, while Hertwig claimed that a tooth arose only after a thickened cone or ledge of epithelium had pushed into the underlying connective tissue. Röse also stated that epithelium was the activating layer in the formation of a tooth.

Since such variation in the interpretation of the development of the mouth in Urodeles exists, Prof. R. G. Harrison suggested to me that further study of the development of the mouth of a typical form, Amblystoma punctatum, particularly by the use of the experimental method, might throw some light on the above-mentioned points. This paper gives the results of regeneration and transplantation experiments employed to analyze the processes which occur during the development of the mouth, the self-differentiating capacity of the stomodaeal ectoderm, and the rôles which the germ layers play in the formation of the derivatives of the mouth. I am glad to express to Doctor Harrison my thanks for material placed at my disposal and particularly my appreciation of his help and interest during the course of this work.

[1] It would be unfortunate to give the impression that workers previous to Gegenbaur had not considered the teeth in Amphibia. Owen, for example, described briefly the development of the teeth in Batrachia, but for the most part he confined his attention to their general distribution, arrangement, and function.

MATERIALS, METHODS, AND NORMAL DEVELOPMENT

For the normal development of the mouth region a series of embryos of Amblystoma punctatum was studied from stage 20[2] with neural folds just closing to stage 46 when the balancers have dropped off and the yolk has been resorbed. These were preserved in corrosive sublimate acetic, embedded in paraffin, cut in sagittal, transverse, or frontal sections of 5 to 10 μ, and stained with Delafield's or Ehrlich's haematoxylin and counterstained with eosin or Congo red. Mallory's triple stain was also found invaluable in stages from 35–46, for it reveals the first traces of dentine in the developing tooth germ.

The method used in the experiments was similar to that outlined by Harrison ('18). In one series of operations, ectoderm was removed from the stomodaeal region of stages varying from 19–30, and the regeneration process was followed. In other series the wound made by lifting the ectoderm from the stomodaeal region was covered with ectoderm taken from the gill region or the ventral body wall of another embryo, homoplastic operations, or in some cases a heteroplastic operation was made by transplanting ectoderm from frog embryos to the wound bed. In addition, the self-differentiating capacity of the ectoderm of the oral region was tested by transplanting it to some other part of the body. Other details of the experiments will be given as it seems necessary. Histological technique similar to that employed with normal animals was used for the experimental series.

A study of the normal stages corroborates the main points in the findings of Kingsley and Thyng, Johnston, and Landacre concerning the formation of the ectodermal collar in Urodeles, and those of Hertwig and Röse concerning the early development of the teeth. The first step in the formation of the mouth occurs in early embryonic history when

[2] The stages are those of the normal plates of Amblystoma punctatum prepared under the direction of Doctor Harrison. These have not yet been published, except a few figures in outline by Harrison ('18) and the ones accompanying the present paper.

ectoderm and endoderm become contiguous. The next step involves the changing of the two-layered ectodermal part of this primitive oral plate into a one-layered condition. This usually begins about stage 25, when the tail bud is appearing, and increases gradually to stages 28 and 29, when the endodermal component of the oral plate shows through the thin, single layer of ectoderm, and also sticks so tightly to the ectodermal layer that it is difficult to remove the latter without disturbing to some extent the tissue just beneath it or surrounding the endodermal plug.

How this one-layered condition of the ectoderm comes about is difficult to decide. Is it, as Johnston suggests, a disintegration of the cells of the inner layer in situ or does the inner layer withdraw until it finally outlines the limits of the endodermal plug? I am inclined to prefer the latter view, as Johnston does, for I have been unable to discover any condition which would suggest the disintegration of the cells in situ, whereas in many specimens the oblique position of some of the cells of the inner layer (fig. 75) suggests either that they are actually withdrawing or that they are passively pushed aside by the outward bulging of the endoderm. It might also be a process involving some phagocytic action on the part of the endodermal cells, but this is doubtful. That the outer layer of the ectoderm disappears completely before the oropharyngeal cavity is established, as Johnston maintains, is unlikely, for its presence can usually be demonstrated in sections.

An examination of the external ventral surface of embryos representing stages 28–42 (figs. 1 to 8) will make clear the main changes in the shape of the oral plate up to the time when the mouth opens. In stage 28 (fig. 1) the plug outline is triangular with the base of the triangle resting against the brain. Between stages 34 and 38 (figs. 2 to 5) a noticeable widening of the base of the triangle occurs, and, in fact, the distinctly triangular outline disappears except that a remnant of the apex remains in the V of the posterior median line. Finally the V disappears (figs. 6 and 7), and

by stage 41 (fig. 7) a thin line across the mouth region indicates the location of the future oral opening. Probably these changes are in some measure due to the shift in position of the mandibular arches which is so evident at this time. During stage 43 (fig. 8) occurs the actual perforation of the oral plate due to the disappearance of the endodermal stomodaeal plug, except for the thin layer lining the mouth. The outer layer of ectoderm also disappears.

A study of sections of embryos in stages corresponding to those just described reveals that the definite boundary of the endodermal plug is due to the arrangement of the inner layer of the ectoderm cells against the endoderm. The cells in the anterior and posterior parts of this border region become less obliquely slanted than in stage 27 and the plug contact is much larger (cf. figs. 75 and 76). At the lateral edges the cells seem to be definitely columnar, prophetic of the ingrowth soon to occur (fig. 77). Over the median portion of the anterior border of the mouth, the hypophysial invagination is active (fig. 11), and by stage 37 it has lost its connection with the external ectoderm and is very closely apposed to the infundibulum of the brain.

After the one-layered ectoderm is established in the oral plate, the next important step is the formation of the ectodermal collar, which has been described in some detail by Kingsley and Thyng, Johnston, and Landacre. About stage 34 (fig. 2), when faint traces of the external gills are appearing, the invagination of ectoderm wrapping around the solid endoderm of the foregut is evident. Growth is most rapid at the lateral borders of the endodermal plug and gradually the hypophysial stalk occupies a relatively much smaller portion of the anterior boundary. The sections of a specimen in stage 35 (fig. 3) give an idea of the extent of the ingrowth of the collar at the edge (fig. 9) where the anterior and posterior components are still united, half-way to the median line (fig. 10) where the components are separated, the posterior one being slightly more extensive than the anterior one, and in the median line (fig. 11) where the

anterior component is lacking due to the presence of the hypophysial stalk and the posterior component is entirely absent. In later stages (36 and 37, fig. 4) the lateral margins of the invaginated areas have extended caudally to a point even with the optic chiasma of the brain and a thin layer of flattened cells from the lateral parts of the collar has grown over the median anterior portion of the gut in the place of the hypophysial stalk. During its ingrowth and after its completion, the collar conforms to the shape of the endodermal part of the foregut and eventually certain of the tooth germs develop in it.

The earliest evidence of actual tooth papillae which I have been able to identify was found in a specimen of Amblystoma punctatum, stage 37 (fig. 4). The papillae were located in the future lower jaw, probably members of the splenial set, and the pulp consisted of three or four cells which appeared to push up freely into the endoderm of the posterior part of the mouth. No indication of a dental ledge appears at this time in the endoderm. This observation agrees with the opinion of Wiedersheim and Röse that the earliest representatives of the teeth in the Urodeles follow the 'placoid type' of development. Neither the anterior (dorsal) nor posterior (ventral) portions of the ectodermal collar give any hint of definitive tooth germs, although the slight thickening along the posterior rim of the anterior component of the collar is suggestive of an incipient tooth ledge. Moreover, no visible indication of developing tooth germs is to be seen in the endoderm continuous with the anterior portion of the collar. A more concrete idea of the shape of the collar and the position of the endodermal tooth germs in stage 37 may be gained by reference to figures 12 and 13, which are diagrams[3] of the floor and roof of the mouth, and to figure 22, which represents a sagittal section through a splenial germ of this stage.

[3] It may be well to describe briefly the method which was employed in plotting the diagrams of the distribution of the teeth shown in figures 12 through 21. These represent the successive stages 37, 39, 41, 42, and 45 in the normal de-

In stage 38 (fig. 5), which is about a day older than the one just described, the number of splenial tooth germs well within the endoderm has increased to five pairs, while two pairs of dentary germs have also appeared. The lateral pair of these is wholly within the ectodermal collar, but the median pair is curious in possessing enamel organs of both endoderm and ectoderm (figs. 24, 78). In the roof of the mouth, three pairs of tooth germs lie almost completely within the ectodermal collar, but in one, and occasionally two, of the pairs parts of the bases of the enamel organs on the border of the endoderm may be composed of cells which contain large yolk granules (fig. 80). Study of subsequent stages indicates that these germs belong to the vomerine series, although the proximity of the vomerine and palatine sets and the absence of the bony plates to which the teeth later become attached make identification somewhat difficult in the earlier stages.

Passing next to stage 39, figures 14 and 15 show the continuation of the development already begun in stage 38. A total of four pairs in the dentary and seven pairs in the splenial sets of the lower jaw, together with three pairs of vomerine and four pairs of palatine germs in the upper jaw, is now present. A pair of maxillary tooth germs in a very early stage of development and a fairly distinct maxillary ledge have also appeared, so that by this stage all sets of tooth germs are represented. Figures 23 and 24 will give an idea of the location of the tooth germs in sagittal sections,

velopment. Cross-sections 10 μ thick were projected upon millimeter paper at a magnification of 100, the median plane of the section being fitted to a line drawn on the paper which arbitrarily represented this plane. The width of the section, the base of each tooth germ, the extent of the collar, and width of the gut were marked in the space corresponding to the section. Points of similar structures, e.g., the points of a single tooth germ, were connected and afterward verified by examination of the section with higher magnification than that used for projection. Finally, tracing-paper was placed over the diagram and a circle drawn to represent the location of each germ. A separate graph was prepared for the roof and the floor of the mouth in each stage. In spite of certain limitations, for example, the fact that a tooth in an oblique position in the mouth, such as a splenial, appears to be smaller in the graph than one which projects perpendicularly into the mouth (a maxillary), the figures serve to give a diagrammatic picture of the regional location of the teeth.

while the characteristic appearance of the histological structure of an ectodermal-endodermal enamel organ of a median dentary tooth in stage 39 may be seen in figure 78. An entirely ectodermal enamel organ and a partially ectodermal one of vomerine teeth are represented in figures 79 and 80, respectively. In addition, an entirely endodermal enamel organ of a palatine tooth germ, the type also belonging to a splenial tooth, is shown in figure 81.

Between stages 39 and 41 (fig. 7), the maxillary tooth germs increase in number and development, and by stage 41 (figs. 16, 17, 25) five pairs of these and a well-developed ledge are prominent features of the most anterior portion of the dorsal ectodermal collar. There is no doubt that these enamel organs are of ectodermal derivation—an observation which is confirmed by the results of the experiments. Moreover, they develop within a dental ledge. Also there has been a noticeable increase in the numbers of the vomeropalatine series (fig. 16), so that they now total fourteen pairs as compared with seven pairs in stage 39 (fig. 14). In the lower jaw (fig. 17) there are five pairs of dentary and twelve pairs of splenial tooth germs. Dentine is present in the majority of these and the earliest representatives of each series bear enamel tips. The beginnings of the bony network which will form the plates to which the teeth become attached have also appeared. Stages 42 and 45 are characterized by a further advance in the differentiation of the germs and some increase in their numbers (figs. 18 to 21). By stage 45 the pointed tips of the oldest teeth have pierced the endodermal lining of the mouth and extend into the mouth cavity which is widely open to the outside. The general position of the ectodermal collar and its boundaries also changes somewhat during stages 41, 42, and 45. In stage 41 (fig. 7) the whole collar, especially its dorsal portion, inclines posteriorly at a rather large acute angle (fig. 25), while at stage 42 (figs. 26, 27), and more noticeably at stage 45 (fig. 28), the angle is small and the bend present heretofore in the gut is effaced. This seems to be in part due to the straighten-

ing of the flexures of the brain and to the greater development of the mandibular arches as well as to the shift in their position. During these stages the posterior limits of the ectodermal collar also become less and less plain as the large yolk granules of the endoderm are used up.

The survey of these stages in the development of the teeth indicates that there is a definite chronological sequence in the appearance of the particular tooth germs. The first germs to appear belong to the splenial set; next come certain of the vomeropalatine series; then the succession returns to the lower jaw with the development of some of the dentary germs, and finally the appearance of the maxillary group completes the sequence. It seems as if the germs of ectodermal origin were delayed in their development due to the adjustment demanded by the thick endodermal plug in the region in which the stomodaeum usually forms. In other groups, notably some selachians and teleosts, the most anteriorly located germs, ones of undoubted ectodermal origin, are the first to appear. On the other hand, in the Dipnoi, which exhibit marked similarities to the Urodeles, the tooth succession parallels that in Urodeles and appears to be an adaptation to similar conditions in that the Dipnoi also possess a thick endodermal component in the oral plate.

Another point, significant because of the relation it bears to the ectodermal collar, is the establishment of the oronasal connection. At stage 40 (fig. 6), the vomeronasal organ is a distinct pouch from the nasal sac, and due to its formation the space between the nasal sac and the roof of the mouth is considerably lessened. About this time a projection, which will eventually connect with the mouth cavity, originates from the nasal sac, lateral to the vomeronasal organ. Moreover, in the roof of the mouth just anterior and lateral to the vomerine teeth, the ectodermal collar is very thin and the endoderm of this region seems to be pushing toward the nasal sac making quite a sharp angle in this area (fig. 25, *ONP*). During stages 41 and 42 the thin ectodermal collar vanishes from this spot and the endodermal cells extend

into the projection which has connected with the nasal sac. Finally, these endodermal cells and the blind end of the projection from the nasal sac disappear and the open passageway is established. This opening usually appears about stage 41, thus preceding the completion of the mouth opening by one or two days.

With this summary of the stages in the normal development of the collar and tooth germs as an interpretative background, the results obtained from experiments may next be recorded.

EXPERIMENTAL

In the experiments on Amblystoma punctatum embryos in which the regeneration process was studied, the stomodaeal ectoderm was removed in stages of development which fall into three main groups: StI, operated in stages 19–23, i.e., during or just after closure of neural folds; StII, operated in stages 24–25, when the tail bud first appears, and StIII, operated in stages 27–30. In some of the experiments the tissue covering the endodermal plug plus a small border, cut 1 (fig. 29), was lifted from the wound bed. In other experiments larger amounts of ectoderm were removed by cuts designated 1+ or 2 (fig. 29), the latter reaching further anteriorly than the former. In these cuts the wound extended laterally well out on the mandibular arch, uncovered the tip of the brain, and overlapped the posterior border of the stomodaeal plug. Animals were preserved at intervals after the operations, in order to obtain series which would show the effects of the operations at various stages of development. The larvae were not kept beyond stage 46, when feeding begins, because by this time the structures of the mouth are established in a definitive fashion and the abnormality of the mouths in many cases made feeding impossible.

A. Regeneration of stomodaeal ectoderm (regeneration or St series)

Establishment of the one-layered condition of the ectoderm in the oral plate. An examination of the material, both before and after sectioning, reveals an agreement in the healing process which occurs in the entire regeneration series. After the cut is made the wound spreads noticeably, and then the tissues contract so that within an hour the wound is closely drawn together with the cells immediately around it much elongated. At the end of twenty-four hours, the majority of the operated specimens seem to be entirely healed, although sections of certain animals fixed twenty-four, forty-eight, and sixty hours after the operation show that complete healing may be delayed for some time. If the wound remains open three days or longer, the specimen rarely lives. During the healing the cells of the external layer of the two-layered ectoderm around the entire border of the wound send out protoplasmic projections in contact with the underlying tissues, endoderm or mesoderm (fig. 82). That cell movement plays a larger part in the closing of the wound than cell division is suggested by the extreme thinness of these cellular processes and of the covering layer and also by the absence of mitotic figures during the first stages of healing.

An increased flexure of the head on the body is apparent externally and even more evident internally. In the majority of the cases the brain is bent into the anterior wall of the foregut so that the latter buckles posteriorly to a greater or less degree into the gut cavity (fig. 30). This causes a variation in the outline of the gut cavity, which in an extreme case may be reduced to a crescentic slit with the anterior and posterior walls of the gut almost in contact. It is probable that the persistence of this flexure is an important factor in the arrangement of the tissue of the gut in more advanced stages of development and may modify somewhat the final result of the operation.

In the regeneration series the covering of the surface of the wound by the outer layer of ectoderm is not followed by the formation of the inner layer in those regions which are in contact with the endoderm. This might be expected because the wound surface is usually covered by one layer of ectoderm at approximately the same stage of development (25–29) or slightly later than that at which it would normally be established, depending on the stage used for the operation. However, specimens in which the ectodermal-endodermal contact is much smaller than the original wound area have two layers of ectoderm around the one-layered area and those in which no contact occurs invariably have the double layer over the entire mouth region (figs. 30, 37, 39). Two possible explanations may be offered for the latter condition: either the contact between ectoderm and endoderm was so slight that it was subsequently destroyed by withdrawal of the endodermal cells or by active migration of the mesoderm into this region; or at the time of the operation the endodermal cells contracted to such a degree that the mesoderm migrated freely over the blunt end of the gut and formed the substratum on which the ectodermal covering developed. The evidence is in favor of the second of these two explanations. Sectioned material of 'no contact' specimens in early and late stages always reveals the presence of mesoderm between the bluntly rounded end of the gut and the ectoderm. Moreover, the records indicate that a plug once visible from an examination of the external surface of the living animal is prophetic of an oral opening in a later stage. While this is not a direct proof, it suggests that if the ectoderm and endoderm come into contact the union is not likely to be severed.

Another fact concerning the one-layered condition of the ectoderm in this series may be seen in a comparison of normal and operated stages. In the normal stages when the one-layered ectoderm of the oral plate is well established (stage 28–29), the outside of the animal shows a plug outline of quite definite shape and size (fig. 1) and the inside a distinct

border-line of cells belonging to the inner layer of ectoderm close to the endoderm (figs. 76 and 77). The ventral external aspect of an operated specimen, however, always differs from the normal in shape, size, and distinctness of the plug outline, and internally in the flatness of the cells of the inner layer around the endodermal plug. As the establishment of this definite border seems essential to the subsequent formation of the ectodermal collar, its prevailing absence in the animals of this series may be in part accountable for imperfect collars.

The method of formation of the one-layered condition of the ectoderm in normal and operated specimens makes this imperfection almost a necessary corollary of the operation. Normally, the one layer comes about by a withdrawal of the inner layer of cells, while here it is dependent on the shape of the area over which the regenerating ectoderm spreads. Thus, there is very slight possibility that a one-layered contact would ever be established which would coincide in shape and size with that of the plug outline usually found in the corresponding normal stage. Consequently, the open mouths formed in operated specimens, which are always correlated with the shape and size of the early plug area, are without exception smaller than the normal and variable in shape.

The formation of the ectodermal collar. In the operated specimens, stages 25–33, the formation of a border of definite shape and character is somewhat delayed, and in the majority of cases is probably never completely attained. Consequently, one would expect to find retarded and imperfectly formed collars in these specimens. In some cases, delay seems to be the most noticeable effect following the operation. The collar is short compared with that of a normal specimen, or is thin, consists of flattened cells and abuts against endoderm which has developed at an approximately normal rate. A comparison of figure 31 of an operated specimen with figure 23 of a corresponding normal stage will indicate the condition above described. Specimens such as these may be cited as evidence that the collar is an ectodermal

invagination and not a transformation of endoderm cells in situ (Kerr). If it were merely a layer of transformed endoderm, there would be in such a case no adequate reason for an imperfect collar.

Variation in the shapes of collars also occurs in many of the specimens. The anterior part is often better developed than the posterior (fig. 32), possibly due to the greater difficulty of healing in the posterior part of the wound—very thin ectoderm exists here at the time of operation—or possibly dependent on some irregularity in the distribution of the tissue of the mandibular arches. Moreover, this inequality of development in different sections of the anterior or posterior component of the collar may be noted. Specimen St20a shows this condition in the anterior component, in which on the right side of the collar there is a bulb-like thickening (fig. 33), a few sections later an indentation of the collar enclosing some mesectoderm cells (fig. 34), while on the left side a well-formed collar exists (fig. 35).

It is also true that the stomodaeal plugs of operated specimens are consistently narrower than those of normal animals. This narrowing seems to be due to the failure of the endoderm to widen laterally. Consequently, the ectodermal collar is narrow, for in its invagination it can only conform to the shape of the anterior end of the foregut. The maladjustment may have its begining in a contraction of the endoderm during closure of the wound and the subsequent failure to establish an oral plate of normal shape and size. Thus a persistent defect in the shape of the anterior end of the foregut would allow mesoderm and mesectoderm, which are in active growth and migration at the time of the operation, to set up abnormal relationships, and these in turn, when once formed, would act to prevent later attempts at regulation on the part of the endoderm. Whatever the primary cause of the difficulty may be, it is a fact that in later stages the lateral extensions of the mouth cavity are often lacking, and consequently the tooth germs of the roof and floor of the mouth are nearly continuous (figs. 38, 42).

Hypophysis. A by-product of this operation is its effect on the hypophysis. Since the cut in every case extends somewhat anterior to the actual boundary of the oral plate, a part or the whole of the hypophysial anlage is necessarily removed. On this point the results of the operations are somewhat at variance, as may be seen from an analysis of the hypophysial conditions in forty-five embryos representing stages 40–46. In this total there are twenty-three specimens which possess an hypophysis, always reduced in size, four with groups of cells which are probably hypophysial and eighteen which show no trace of hypophysial ingrowth. Moreover, of the ten specimens representing cut 2 in group StIII, operated in stages 27–30, nine have no hypophysis, while of the ten representing cut 1+, only two lack an hypophysis and the others possess definite traces of hypophysial cells with the possible exception of one animal. It is also significant that in the four specimens in which no mouth has formed owing to the absence of an oral plate, there is no definite hypophysial structure.

Formation of tooth germs. The formation of the tooth germs in the ectoderm of the operated specimens, as in the normal, is obviously dependent on the formation of the collar, and the conditions which are found here may be in large measure understood by keeping in mind the modifications which appeared in the invaginated ectoderm. On the other hand, the splenial and palatine tooth germs which are located in the endoderm are rather constant in their time of appearance and to some degree in their arrangement, oftentimes being quite similar to the normal condition (figs. 31 and 36). It is true, however, that variation in the shape of the foregut due either to buckling of its anterior wall posteriorly, or to imperfect relations with the mandibular arch tissue, or to lack of contact with the external ectoderm, will have a distinct and to some extent a characteristic effect on the position of these tooth germs. In one specimen with an extreme flexure the palatine teeth are pushed behind the splenial series while in figure 37 may be seen the usual grouping of the palatine

and splenial tooth germs over the lateral boundaries of the anterior end of the foregut in individuals in which the oral plates are absent. The massing of these germs often occurs also in individuals in which the lateral extensions of the mouth on one or both sides are lacking. This may be seen in figure 38, where on the left side the palatine and splenial tooth germs are well separated by an approximately normal extension of the mouth, while on the right the two groups are in close proximity, even having their basal bony plates nearly fused. Again, in figure 42 the width of the gut is much reduced and the splenial tooth germs are almost dorsal to the gut on both sides.

If the derivatives of the ectodermal collar are next examined, it is found that in many of the operated specimens (except those in which no collar is formed due to lack of ectodermal-endodermal contact) representatives of all of the sets of teeth may be present. The vomerine and maxillary tooth germs are the most constant in their formation—a fact probably correlated with the more normal development of the anterior component of the collar. The derivatives of the posterior part of the collar, the majority of the dentary germs, are, however, markedly variable—a fact which seems to bear some relation to the less perfect ectodermal ingrowth in this region.

Although the vomerine and maxillary tooth germs are generally represented, they are often retarded in formation as the collar was. For example, only one pair of well-developed vomerine tooth germs or occasionally a single germ is found in an operated specimen at stage 38, although normally three well-defined pairs should be present. In stage 40, regularly possessing three pairs of maxillary tooth germs, a maxillary ledge may be the sole indication of dental development in this region. In other cases the delay is slight. A section (fig. 36) from specimen St37a, stage 40, shows in the ectoderm a moderately well-formed median maxillary tooth germ, together with the tips of a vomerine and a dentary germ, as well as two splenial tooth germs in the endo-

derm. In the remaining sections of this animal, three maxillary and two vomerine teeth and a vomerine ledge are present on each side, while the dentary group is represented by two fairly distinct germs lodged mostly in the endoderm and traces of at least two other pairs in the ectoderm. In another case the unequal development within the anterior component is evident, in that on the right side, vomerine, palatine, and splenial germs are represented, while on the left only members of the palatine and splenial groups are to be found.

The most important contrast in development is to be seen in the lack of maxillary, vomerine, and dentary tooth germs in specimens with no ectodermal-endodermal contact (fig. 37). It is barely possible in such specimens or those with a minute contact that the median pair of dentary germs, which normally possesses enamel organs composed of both ectodermal and endodermal cells, may be formed totally from endoderm even if no collar is present. This is occasionally suggested by finding two germs whose position appears to be that normally occupied by this pair of teeth (fig. 41), but there are no individual characteristics of the dentary group which would serve as a means of identifying them with absolute certainty.

In several cases in which no mouth opening has been formed, or in which only a minute one is present in the posterior portion of the mouth region, a structure occurs which I have termed a 'maxillary bulb.' These bulbs are small invaginations of ectoderm, which in a late stage usually contain one or possibly two rather well-developed tooth germs and are completely isolated from any contact with the gut endoderm (fig. 39). If but one bulb occurs, it may be found either on the right or the left side or in the median line (fig. 40). Occasionally one is present on each side. From this formation one would conclude that the ectoderm must possess some potentiality for independent invagination without endodermal contact, because examination of the sections of these bulbs reveals no trace of endodermal cells with large yolk granules, nor in later stages any space from which the endoderm might have disappeared. These bulbs also suggest

that the formation of a tooth is dependent in certain cases on an interaction of the ectoderm and mesectoderm in a specific region.

In summarizing the development of tooth germs which has occurred in the regeneration series, one may state that all of the sets, maxillary, vomerine, palatine, dentary, and splenial, may be found. This is true regardless of the stage at which the operation was performed and to some extent of the cut used. The time of development and location of the palatine and splenial groups, which have endodermal enamel organs, are the most constant. Those possessing enamel organs of ectodermal composition are nearly normal, although generally retarded, massed, or absent in accordance, respectively, with the degree of perfection attained by the collar in its development or its total absence.

An analysis of conditions preceding the development of the collar shows that its formation is dependent on the extent of the contact made by the regenerating ectoderm with the underlying endoderm. The permanence of the flexure which occurs at the time of healing and the subsequent developmental relations of the mandibular arches also play no small part in this process. It seems to be true that the formation of the mouth—shape, size, and derivatives—is the result of an interaction between the three germ layers of which the endodermal tissue of the gut probably forms the keystone. The shape of the endodermal gut following the operation appears to be an extremely variable factor. Its cells must contract rather markedly in cases of complete withdrawal from ectodermal contact, thus decreasing the gut cavity. Perhaps this contraction, always present to some degree, together with the effect of the flexure, may be responsible for the irregular distribution of the mesectodermal anlagen of the meckelian cartilages.

Formation of the mouth. Although I have referred to the necessity for an ectodermal-endodermal contact as a preliminary for the formation of the open mouth and the correlation of the shape and size of the plug in early stages with the

shape and size of the mouth in later stages, an example will illustrate how close this correlation is. In three conspicuous cases, the records of the individual specimens state that each of them possessed a split plug at an early stage, and in later stages the mouth opening in each consisted of two minute holes (fig. 43). Similarly, a plug located on either the right or the left side or in the median line is prophetic of an opening which will be correspondingly situated. These facts together with the absence of an opening when the record states that 'no plug is present' furnish proof of the necessity of the early establishment of an ectodermal-endodermal contact for the development of a future mouth opening. Such points and a consideration of the nature of the operation help to explain the rarity of even partially perfect mouth openings.

Oronasal openings. Usually within a day of the appearance of a mouth opening occurs the perforation of the oronasal tubes in normal specimens. Hinsberg ('01) states that the posterior choanae in Triton are situated in the endoderm, while Johnston locates these openings in Amblystoma punctatum in the region of the ectodermal collar, slightly anterior and lateral to the vomerine teeth. The results of these experiments seem to uphold Johnston's opinion, in so far as they indicate that correct topographical relations between nasal sac and roof of the mouth, particularly ectodermal collar, are probably essential for perfect oronasal connections. Those cases in which no oral plate is present are possibly too extreme to be regarded as more than suggestive of this fact, in that the amount of separation of the gut region from the sac is so great that the chance of a contact approaches zero. In other cases, although an ectodermal collar is present, there is a marked reduction in the lateral extensions of the mouth, and again the mechanical difficulty of separation arises in the way of establishing the contact between nasal sac and mouth.

In still other instances an actual contact of the nasal sac and gut may exist, but no perforation opening up a free

passageway from one into the other occurs. This is especially surprising in specimens in which on one side the nasal sac opens into the gut cavity, while on the other side it does not, although it would appear that all the factors—nasal sac, ectodermal collar, and gut endoderm—which are thought to be essential for the connection, are present on both sides. This makes it seem likely that a relationship exists which is hard to detect and even more difficult to analyze. In one individual it is possible that a few mesectodermal cells may have become wedged between the nasal sac and the mouth cavity and may be forming an effective barrier to the perforation.

Shape of head. To the data which concern the mouth derivatives, it may be well to add a brief description of the shape of the head in operated specimens of the regeneration series. As a general rule, the disposition of the structures in the head bears a rather close resemblance to the normal condition (fig. 53), the greatest variation being found in the shape of the mouth. Occasionally (fig. 51) an animal has the characteristic small head and protruding mandibular-hyoid region of the transplantation series, but this condition is found only in specimens which lack an ectodermal-endodermal contact or in which only a very small mouth opening is present. Even in cases in which a fairly extensive contact area is located asymmetrically on the right or left side, the shape of the head may be approximately normal. Internally, however, the structures, especially the cartilages, may be abnormally arranged. In one specimen with a very small opening the two meckelian cartilages are situated high at the sides of the foregut (fig. 41). In other instances one meckelian cartilage is located laterally or almost dorsally to the gut, while the other possesses nearly normal topographical relations (figs. 38 and 42). If the plug contact or mouth opening is lateral to the median line, the bar on the side without the contact or opening is invariably more abnormal in shape and position than the other. In the extreme 'no contact' cases the meckelian cartilages may be repre-

sented by a single thickened bar immediately in front of the foregut wall. Such a bar bears slight trace of its derivation from two lateral mesectodermal anlagen.

Comparison of the groups in the regeneration series according to stage and cut used in operation. A comparison of the results of the operations, grouped according to the stage of development used for the operation and the cut employed, is suggestive. Three groups of animals were used for these experiments, namely, StI, operated in stages 19–23; StII, operated in stages 24–25, and StIII, operated in stages 27–30. The cuts were also three in number, designated 1, 1+, and 2 (fig. 29). Regeneration following cut 1 in an early stage of development is most successful. Five openings, quite similar in shape to the normal condition, although somewhat smaller, and two with small lateral openings, each of which has one fairly well-developed lip fold, make up this group. Following the use of cut 1+ in the StI group, of seven animals in stages normally possessing mouths, three resemble the normal condition except in the size of the mouth (fig. 44). Of the four remaining specimens three have small openings on either right (fig. 46) or left side (fig. 45), and one possesses two minute holes near the median line (fig. 43). Thus it would seem that the relatively small amount of tissue removed in cut 1 can be compensated more adequately than the larger amount in cut 1+.

In group StII, seven animals belonging to a series in which cut 1+ was employed have reached the stage when the mouth opens. Of these, two individuals show small median openings (figs. 47 and 48), even smaller than in group I, while two others have very small perforations located on the right side (fig. 49) and three present no trace of a mouth opening (figs. 50 and 51). These facts seem to indicate that postoperative adjustment during stages 24–25 is more difficult than during stages 19–23.

In group StIII, operated in stages 27–30, there are fourteen specimens representing cut 1+ and five specimens representing cut 2 in stages of development 42–46. All of the fourteen

of cut 1+ have developed well-shaped oral openings (fig. 52), varying in size from 0.3 to 0.8 mm. Of the five specimens of cut 2, three have moderate-sized openings, while two show barely visible clefts. Here the significant comparison is between the 1+ group of this series and the corresponding specimens in StI and StII and the former in contrast to those of the latter group have mouths rather normally shaped.

The conclusion suggested by these data is that the process of recovery and adjustment during stages 27–30 is more perfect than during stages 19–23 or 24–25. This may be due to the fact that differentiation has proceeded to such a degree that the mesodermal cores and the mesectoderm of the mandibular arches at this time are well formed and in their normal position. In such cases the removal of stomodaeal ectoderm and its border region does not so markedly disturb a pattern, which is rather well established, and recovery does not involve such thoroughgoing adjustment of tissue relations. It is true, also, that during these stages the shape of the anterior end of the foregut is becoming 'set' in its relation to the external ectoderm and to the mandibular arch tissue. The successful results in StI may be explained by the fact that the tissues recover quickly in early stages. In the intermediate group, on the other hand, active changes are taking place in connection with the formation of the arches and migration of mesectodermal cells. Consequently, the need for adjustment is great and many difficulties are encountered. These conclusions, however, must be tentative until additional data can be secured from a larger number of experiments.

B. Transplantation experiments

The transplantation experiments consisted of the removal of the stomodaeal ectoderm in stages 21–29 by cut 1+ or cut 2 (fig. 29) and its replacement by ectoderm taken from the gill region (StG series) or from the ventral body wall (StV series) of another specimen of the same age and species, thus making a homoplastic graft. Also in certain cases heteroplastic grafts were made by covering the wounds with ecto-

derm from frog embryos. In addition, reciprocals of the homoplastic type were represented by experiments in which the stomodaeal ectoderm was grafted to the gill region of another specimen (GSt series) in an attempt to determine the specificity of the cells of the transplant.

In the homoplastic operations the donor animals were stained with Nile-blue sulphate (Detwiler), thereby making it possible to determine the extent of the graft even in a late stage. Occasionally a few mesoderm cells stuck to the gill ectoderm, but this was recorded in every case and did not materially affect the results of the operation. The orientation of the graft was also varied, but this, too, seemed to cause no noticeable effect in the results. The animals were kept in a cool chamber (14° to 17°C.) until the establishment of the heart beat, and although this procedure slowed the development somewhat, it lowered the mortality rate considerably.

1. Transplantation of homoplastic gill ectoderm to the stomodaeal region (StG series). This group of experiments is represented by 121 operations, for each of which an individual record was kept. Forty per cent of the animals died owing either to imperfect healing in early stages or to the difficulty experienced by the animal in establishing a normal circulation in later stages. The remaining specimens, seventy-three in number, were preserved in stages from 28–46 and either sectioned or dissected for study.

a. External appearance of the animals. The external appearance of these animals differs quite noticeably from that of the specimens representing the regeneration series. The flexure of the head on the body is much less acute, probably because the insertion of tissue makes the contraction during healing less severe than in the regeneration series, where the closure of the wound is entirely dependent on the animal's own tissue. A flattening of the mandibular arches is apparent in the majority of these cases (cf. figs. 54 and 55) and is indicative of far-reaching effects on the process of the formation of the head in this group of experiments.

However, the contours of the animals in later stages are perhaps their most characteristic feature. These may be seen in the photographs (figs. 72 to 74) of specimen StG51 and its normal control. In the dorsal view (fig. 72) it is evident that the head of the operated animal (to the right) is narrower and more pointed than that of the normal one (left). The most extreme contrast, however, is that in the profile view. The anterior portion of the head of the operated specimen appears short in its dorsoventral axis, while the hyoid and branchial regions project conspicuously and the operculum is always ballooned. It is clear that an excessive massing of tissue has occurred in this region. On the ventral surface, the projecting mandibular and hyoid region is usually rounded at its anterior edge and the grafted tissue in the area just in front of the projection slopes backward gradually to the ventral portion of the operculum.

The extent of the graft in these specimens is rather constant, as would be expected, since a standard cut was made in the entire series. In a late stage, the transplanted tissue generally masks the ventral surface of the head, reaching anteriorly on a level with the nasal sacs and often forward between them for some distance. Its lateral boundaries then slope backward in line with the bases of the balancers and sometimes the stained cells may be found covering the ventral surface of the balancers. The posterior boundary may be scalloped or rather straight and the tissue may extend a greater or less distance toward the heart. Usually small hillocks appear on one or more regions of the grafted tissue, and in a few cases a projection, suggestive of a gill filament, extends out from its surface for a brief period.

Another feature which is obvious from an examination of the living animals is that many of the operated specimens are 'pale' when compared with their normal controls. This fact suggested at once that on removing the stomodaeal ectoderm, the hypophysial anlage had been wholly or in part extirpated, and this is corroborated by study of sectioned material. This point will be considered later.

b. Establishment on the one-layered condition of ectoderm in the oral plate. When the wound is healed, it is comparatively easy to locate the boundaries of the stained grafted ectoderm in the living state, and even in sections of material preserved in early stages the line of union may often be identified owing to the contrast between the thick gill ectoderm and the thinner ventral ectoderm. Since the gill ectoderm is two-layered, the problem of establishing a one-layered condition is similar to that in the normal animal. Here, as might be anticipated from the normal development and from the results of the regeneration series, the appearance and extent of the one layer of ectoderm in the oral plate is dependent on the presence and size of the contact between the grafted ectoderm and the endoderm of the gut, and in those cases in which no union occurs the two-layered ectoderm persists. This establishment of a one-layered condition in the foreign ectoderm suggests that endoderm plays an active part in causing the withdrawal of the inner layer of the ectoderm. A priori, there is no reason for the inner layer to disappear, as might be true of the ectoderm normally located here and possibly possessing some potentiality for this particular change.

In normal development, when the one-layered condition is well established, the inner layer of ectoderm forms a distinct border around the endodermal plug. In the transplantation, as in the regeneration series, the distinct border is seldom well developed. Instead, the inner layer of ectoderm tends to shade off obliquely at the borders of the plug, although occasionally a rather definite boundary will be established at one spot while the rest of the outline may be indistinct. One fact must be emphasized in connection with the contact area. In none of the specimens does the external appearance of the endodermal plug in contact with the ectoderm ever approach the normal plug outline of the corresponding stage or even the imperfect condition of many of the plugs in the regeneration series.

Furthermore, lack of contact between the endodermal plug of the foregut and the graft is always correlated with a per-

sistence of the two-layered condition of the foreign ectoderm and in later stages results in an absence of the mouth opening. It would be of interest to know the cause of this lack of union on the part of these two germ layers, because the condition is very prevalent in this series of operations. Of sixty-four animals sectioned, forty of them (62.5 per cent) showed a two-layered condition of the ectoderm and no connection between the gut and the ectoderm. This condition may be due to a contraction of the gut endoderm at the time of the operation, as was suggested for similar cases in the regeneration series, reducing the chance of a contact between ectoderm and endoderm. Or, following the removal of the stomodaeal ectoderm, a flexure may cause a buckling on the part of both brain and gut endoderm. If either or both of these processes occurred, there would be ample opportunity for the migration of mesoderm and mesectoderm into the space and the gut and the ectoderm would be permanently separated from each other.

c. The formation of the collar and the development of tooth germs in specimens with an ectodermal-endodermal contact. As might be expected from the study of the regeneration series, only those animals in the transplantation group which possess an oral plate of one layer of ectoderm and an endodermal component develop ectodermal collars and their derivatives, namely, the maxillary, vomerine, and dentary tooth germs. There are, however, variations in the condition of the ectodermal collars and the tooth germs developing from them, although it is always true that the collar conforms to the shape of the endodermal foregut. In certain of the collars formed, typically arranged columnar cells are found and the invagination of the collar approximates that of a normal specimen (cf. figs. 56, 57, 58 with figs. 23 and 24). Also in some specimens good dentary, vomerine, and maxillary teeth and their ledges may be present. Figure 57 illustrates the position of a vomerine tooth in one of these individuals. In another case, however, no dentary tooth germs are visible and the entire posterior portion of the collar is poorly developed,

while in the anterior portion of the collar of this specimen maxillary and vomerine tooth germs are found in a condition somewhat similar to the normal state.

In other cases, which may be termed 'doubtful collars,' the condition is not so clear-cut. Part of the difficulty of interpretation lies in the fact that an ectodermal invagination appears to have taken place, but no well-formed tooth germs are present in the ingrowth. This is true at a stage of development when the ectoderm should contain several pairs of germs in both roof and floor of the mouth. Another difficulty encountered particularly in late stages of development, 44–45, is that the criterion for distinguishing ectodermal from endodermal cells, namely, the presence of large yolk granules, has nearly disappeared from the anterior endoderm. Specimen StG52 (fig. 59) illustrates such a case as that just described. A very limited connection of the gut with the ectoderm is present, and although there is evidence of some ectodermal invagination, no sharp line of demarcation exists. In specimen StG116 (fig. 60) the contact covers a larger area, but the limits of the ectoderm and endoderm are not clear due to the disappearance of the large yolk granules.

These cases in which tooth germs with ectodermal enamel organs are not present in the invaginated tissue raise the question as to whether this grafted ectoderm has the potentiality of forming tooth germs in its new position. They seem to argue against this possibility. It must be noted, however, that in these cases the plug and therefore the collar is very small and abnormally shaped and the invagination is located approximately in the median line of the animal in a region which normally possesses few tooth germs, as may be seen in the diagram showing the distribution of teeth in stage 45 (figs. 20 and 21). Thus the absence of tooth germs in these cases does not wholly decide the question.

On the other hand, there are the few cases above described in which collars with representatives of all or some of the ectodermal tooth germs are present. These seem to offer no possibility of any other explanation than that the foreign

ectoderm has invaginated and with the mesectoderm of the region has given rise to tooth germs similar to normal ones. These specimens were examined very carefully on the suspicion that the host's own tissue might have had an opportunity to invaginate and to form the collar and the tooth germs. In one individual the sketch of the ventral view in an early stage suggested that this possibility might exist, but the junction of the foreign and host tissue showed plainly in the sections and it was evident that the grafted tissue had invaginated. It is true, also, that the collars which contain good tooth germs have a fairly large lateral extent, in contrast to the small medianly located collars of the animals which lack ectodermal teeth. Thus, these specimens would seem to give a positive answer to the question as to the ability of the foreign graft to form teeth and also suggest that the mesectoderm may have a more prominent part in instigating this formation than is usually ascribed to it. Furthermore, the relation of this foreign ectoderm to the endoderm over which it has invaginated may have some part to play in the process.

To the foregoing may be added a description of the condition of the tooth germs in the endoderm of this transplantation series. The constancy of their appearance is as striking as it was in the regeneration series. If the collar is well-formed both as regards size and shape, or better if the anterior end of the foregut approximates the normal condition—a rare happening—the arrangement of these teeth approaches the normal (figs. 58 and 60). This is not surprising when one considers that the factor which holds the endoderm in its normal position is its union with the ectoderm. As has already been seen in the regeneration series, lack of contact is followed by a massing of the endoderm which results in a more or less close union of the anlagen of the splenial and palatine tooth germs (figs. 62 to 64).

In addition to true collars which invaginate in association with the endoderm of the gut, in some specimens there are also found independent invaginations of ectoderm which give

rise to tooth germs similar to the 'maxillary bulbs' of the regeneration series. They are generally present near the nasal-sac region and seldom exhibit any connection between their tissue and that of the gut (fig. 61). It is interesting also to find in rather early stages, before tooth germs are formed, small ectodermal invaginations which are possibly 'incipient bulbs,' although they cannot be identified with certainty. One possible point of difference between these 'bulbs' and those of the regeneration series is that here the question arises as to the origin of these structures, whether from foreign or host tissue, while in the regeneration series there is no alternative. A comparison of the location of the bulb in sectioned material with the extent of the grafted ectoderm as seen on sketches of the animals seems to indicate that the grafted ectoderm is responsible for the bulbs of certain of the specimens. This is true in specimens StG108 and StG116 (fig. 60). In the other cases in which I have found such structures they have been located in the transition region between graft and host tissue, and the junction between the two tissues did not show plainly enough to make a decision regarding the origin of the bulb possible.

d. Conditions in specimens with no ectodermal-endodermal contact. In this transplantation series the prevalence of the condition in which an ectodermal-endodermal contact is lacking stands out emphatically. Here, as in the regeneration series, which rarely showed this condition, may be found clear-cut evidence that an ectodermal collar is essential for the production of maxillary, vomerine, and dentary tooth germs, because these are wholly absent in such specimens. In the early stages of the 'no contact' state there is always a separation of ectodermal and endodermal tissue either by loose mesenchyme or more often by compact mesoderm and mesectoderm. The blind end of the foregut may be of two distinct types, according to the degree of separation of its dorsal and ventral walls. In one type the endoderm is a relatively short distance from the external ectoderm and the palatine and splenial teeth are nearly normal in their dis-

tribution. Such a case blends into the 'contact' type, in which an ectodermal-endodermal contact may exist over five to ten sections (fig. 59). On the other hand, the separation may be so slight as to be indicated in sagittal sections only by a small V-shaped indentation between the palatine and splenial tooth germs (fig. 62). Even within the second group, in which there is no separation of dorsal and ventral walls of the mouth cavity at the anterior end of the gut, there exist differences of condition according to the amount of massing which has occurred in the endoderm. For example, in a sharply truncated gut the palatine and splenial teeth, standing in a line perpendicular to the long axis of the body, are still separated by some expanse of tissue (fig. 63), while in another case palatine and splenial teeth are so closely grouped that identification of individual tooth germs is somewhat uncertain (fig. 64).

In late stages of normal development the splenial teeth become anchylosed by a bony network to the posterior median surface of the meckelian cartilages, while the dentary tooth germs become attached to the anterior section of these cartilages. A similar condition exists in the operated animals and aids particularly in identifying the splenial tooth germs. The question naturally arises as to the interdependence of the mesectoderm which forms the pulp of the tooth germs and the meckelian cartilages and the endoderm which gives rise to the only enamel organs present (in 'no contact' cases). The study of animals in normal stages and in regeneration and transplantation experiments confirms the findings of Platt, Landacre, and Stone ('22) in respect to the general distribution of the mesectoderm in Urodeles, and further seems to indicate that its migration is molded on the mesodermal cores of the arches and the shape of these, in turn, is somewhat dependent on the configuration of the anterior end of the foregut. For example, the lateral edges of the anterior foregut and their contact with the ectoderm normally serve as a wedge to separate the migrating neural-crest cells of the trigeminal region into mandibular and maxillary proc-

esses about stage 30. However, in operated specimens the entire absence of the endodermal wedge due to lack of an oral plate allows the material of these processes to remain united and to fill in the space ordinarily occupied by the endoderm of the foregut and in later stages the abnormal position and shape of the meckelian cartilages testify to this early disarrangement of the tissues (figs. 62, 63, 66, 68).

Since this abnormal distribution of the mesectoderm is so apparent, the question arises as to what extent it may influence the position of the tooth germs. Is the indication that the endoderm and not the mesectoderm takes the lead in locating the dental anlagen? A consideration of the data bearing on these questions makes one think that the position of the tooth anlage is more or less determined in the endoderm. (I am barring, for the time being, any discussion of ectodermal tooth germs, for the constancy in the appearance of the endodermal ones makes the problem particularly applicable here.) For example, even in individuals with abnormally shaped guts, especially in 'no contact' cases, the splenial and palatine tooth germs are often identified largely from their endodermal relationships, although even here it is true that mesectoderm forms their pulp and must have been present in this location from a relatively early stage. Thus, whether the endoderm or mesectoderm has started this development is still an open question, and it may be the case that these layers are closely interdependent for tooth formation; or, in other words, even if the endoderm takes the initial step in the formation of a tooth anlage, it may possibly do so because of its interaction with mesectoderm rather than because of its innate potency, and without the presence of mesectoderm it is not certain that the process would begin and even more doubtful that it would continue. However, that some potentiality for the formation of teeth exists in the endoderm of the floor of the gut is suggested from the fact that just posterior to it in the endodermal tissue over the hyoid arch, no such anlagen are found, although the mesectoderm of the hyoid cartilages is at least superficially similar

to that giving rise to the meckelian cartilages and to the papillae of the teeth.

Concomitant with the abnormal development in the meckelian cartilages there is also some irregularity in the hyoid and branchial cartilages. It consists, for the most part, in displacement due to cramping as is suggested by an examination of the profile views of these operated animals (fig. 74). This may also be seen in the outlines which show the ventral and lateral views of dissections of the cartilages in an operated specimen StG74 (figs. 66 and 68) and its normal control (figs. 65 and 67) prepared by the methylene-blue method. It will be noted here that the arrangement of the branchial cartilages is abnormal in contrast to the meckelian cartilages, where shape, size, and location may all be irregular. This indicates that only the anterior end of the foregut suffers a marked modification in shape, while the posterior pharyngeal region and to some extent the chondrocranium are indirectly disturbed by cramping.

Another structure which is somewhat affected by developmental relations in this series of operations is the brain. This appears in a comparison of sagittal sections of normal and operated animals, but it is more evident in figures 69 and 70, which are drawn from dissections of the brains of StG74 (fig. 70) and its control (fig. 69) in stage 45. Here, the anterior end of the brain in the side view of the operated animal is smaller than in the normal specimen and part of this reduction in size seems to be due to the somewhat lateral displacement of the cerebral hemispheres. The cerebellum is also pushed over the fourth ventricle and the infundibulum is developed into an abnormally thin and large sac, hanging down from the rest of the brain (fig. 70). This condition may be due to a lack of interrelationship between the hypophysis and the infundibulum.

e. Hypophysis. Of the sixty-four animals sectioned in this group, the hypophysial invagination is lacking in fifty-two. Only one of these specimens was preserved in a stage before the hypophysis normally develops. Of the eleven which are

classed as uncertain, each shows only a small clump of cells which might be considered hypophysial, and half of these are in early stages when there is some chance of confusing the hypophysis and displaced mesectodermal cells. Only one of the sixty-three animals possesses an hypophysis which can with certainty be identified as such, and this is in a specimen with an ectodermal collar (fig. 56). In sharp contrast to this section is one from StG53 (fig. 63), which shows no trace of any cells which may be hypophysial, although the section is cut in a plane which would normally reveal their presence.

Correlated with this complete absence, it is worth noting that in every case the record of the individual specimen states that the animal was 'pale' when compared with its normal control, and in the sections the melanophores are contracted tightly. This evidence seems therefore to indicate that at stages 44–45 (before the yolk is quite resorbed), and in one case earlier (stage 40), the hypophysis may be exercising some influence on the condition of the melanophores. This is in line with the work of Allen ('16, '17), Atwell ('19), Smith ('16, '19, '20), and Swingle ('21), as to the part which the hypophysis plays in the normal coloration of Anura.

The contrast between the condition of the hypophysis in the regeneration series and in the transplantation series is very marked. Comparison of the results following cut 1+ in both series for specimens in stages 40–46 is given in the following lines:

	Hypophysis present	*Not present*	*Uncertain*
Regeneration series	18	8	4
Transplantation series	0	17	5

This suggests that insertion of foreign tissue has blocked the possibility of regeneration and invagination on the part of any cells of the hypophysial anlage not removed. It may also be true that the lack of ectodermal-endodermal contact may be in part responsible for the non-occurrence of hypophysial invaginations because four of the five doubtful hypophyses are found in individuals which have a definite con-

tact and therefore provide a condition somewhat similar to the normal for the hypophysial ingrowth.

f. Oronasal connections. Other structures which were markedly affected in development following the operation in the regeneration series are similarly disturbed in the transplantation series. These are the oronasal connections, which are consistently absent regardless of the presence or absence of an oral plate. It is not surprising that specimens lacking an ectodermal-endodermal contact and the anterior portion of the mouth cavity also are devoid of oronasal connections. In those with a contact and therefore with a portion of the foregut region present it might be expected that a connection would occur. However, in these cases also, the location of the anterior end of the gut is so far posterior to its normal position, and therefore its distance from the nasal sac so great, that the chance of a contact is exceedingly small. In fact, in only one case in this series was there any evidence of contact between nasal sac and foregut tissue. In this specimen, StG51, stage 45, on the left side of the mouth the nasal sac and gut tissue touch over five sections, but no perforation exists. This case seems as inexplicable as the similar ones described in the St series, because it would seem that all of the factors deemed essential for the formation of this connection, namely, nasal sac and ectodermal collar covering an endodermal column of cells, are present.

Three possible explanations of this condition may be offered. The tissue present may not be a true ectodermal collar. There is small likelihood of this, for close to the point of contact of nasal sac and mouth are to be found teeth which have been identified as belonging to the vomerine series. There also exists the possibility that a few mesectodermal cells may have been caught between the nasal sac and mouth tissues and may be a barrier to any open passageway, but such cells cannot be identified. Finally, it is possible that with the stomodaeal ectoderm a portion of the nasal-sac placode may have been removed (GSt series), and that incomplete regeneration of the nasal sac followed, and therefore no perforation develops.

Summary. In summarizing the data concerning the structures of the mouth in this group of experiments, it may be stated that certain conditions seem characteristic of the series. The most noticeable feature is the lack of an oral plate and the presence of two-layered ectoderm in the mouth region in forty of the sixty specimens which are in stages normally possessing a contact between gut endoderm and the external layer of ectoderm. Correlated with this lack of contact, ectodermal collars are relatively few in number and not all of those in stage 38 and following reveal traces of ectodermal tooth germs. In contrast, the endodermal derivatives, splenial and palatine tooth germs, are constant in their time of appearance, although they may be somewhat abnormal in their arrangement. Furthermore, of nineteen specimens in stages 42–46, only four animals possess open mouths, and these are much reduced in size. Thus, one is led to consider the contact of ectoderm and endoderm in an oral plate as a fundamental requirement for the formation of a normal mouth. And this is true not only as far as the definitive structures of the mouth are concerned, but also for the arrangement of the tissues which surround the anterior end of the foregut.

2. Transplantation of homoplastic ventral body wall ectoderm to stomodaeal region (StV series). In this group of animals ectoderm from the ventral body wall was transferred to the wound (StV series). This ectoderm is thin and it is therefore somewhat difficult to remove the mesoderm from it without injury. Thus the operation involves certain difficulties not encountered in the previous series. One hundred and thirty-one animals were operated upon, and of these, seventy-one were preserved in stages ranging from 33–46. The three cuts designated as 1, 1+, and 2, which were employed in the regeneration series, were also used here. However, since cut 1+ was the one used in the gill-transplantation series, only those animals with that cut or cut 2 in the StV series will be compared. The results of these experiments are in the main in close agreement with those obtained in the other series.

In one significant point there is an apparent difference between this series and the gill-transplantation series. This concerns the potentiality of the ectoderm from the ventral body wall which was used for the grafts. In spite of the fact that the same cut (cut 1+) or a larger one (2) was employed, the records often state that one edge of the plug, either left or right, or sometimes the posterior portion remained uncovered. This condition was noticeable soon after the operation, although at the time that the clamp was removed, the graft seemed to cover the entire plug. In the gill transplants this difficulty was never encountered. It is probable that this contrast is due to a different growth potentiality on the part of the two kinds of ectoderm. While the gill ectoderm is composed of rather thick layers of cells, which in their normal position develop the extensive outpushings of the external gills, the thin ventral ectoderm gives rise only to the skin of the ventral body wall, a smooth, flat surface. Therefore, when the grafts are transferred, it seems not unlikely that the layers of the cells will continue to develop somewhat in accordance with their usual potentialities for growth.

This series also raises the question as to whether ectoderm from the ventral body wall can give rise to tooth germs in its new position. An examination of the cases representing cut 1+ and cut 2, in which a plug showed through the graft and in which, therefore, the collar was apparently formed within the grafted area, does not give any definite answer. In certain of these cases ectodermal enamel organs may be found, but they are generally associated with 'maxillary bulbs' which are so far anterior that they are probably beyond the grafted area. In connection with the opening formed, which is somewhat posterior to its usual position, only tooth papillae belonging to the endodermal groups seem to be present. Occasionally two tooth germs which occupy the position of median dentaries can be identified. In one case only is there evidence of a collar containing maxillary and vomerine tooth germs and a plug outline located within the

graft. This isolated case does not present sufficient evidence to be very convincing except as it appears to confirm the results of the gill-transplantation series.

Another point in which this series agrees closely with the gill-transplantation group is in the fact that here also there is a striking dearth of hypophyses. This absence is more consistently found than in the other series. In nineteen animals representing stages 40–46, in which either cut 1+ or cut 2 was used, only one very small hypophysis has been identified, besides two cases which are very uncertain. Unfortunately, the records fail to describe the coloration of seven of the sixteen animals which show no trace of hypophysis, but an examination of the melanophores in the sectioned material indicates that they are greatly contracted. In the nine remaining animals which lack hypophyses, the melanophores are also contracted and, furthermore, the records state that these specimens were distinctly pale in comparison with their normal controls.

3. Transplantation of heteroplastic tissue from frog embryos to the stomodaeal region. To the homoplastic-graft operations another group of experiments was added, in which ectoderm from three species of frog embryos, Rana pipiens, Rana sylvatica, and Rana palustris, was grafted on the wound bed made by removing the stomodaeal ectoderm from embryos of Amblystoma punctatum. The donors were usually in the high neural-fold stage, and therefore somewhat younger than the Amblystoma embryos, which represented stages 22–27. This difference in age allowed for the more rapid development of the frog tissue. On the whole, these experiments were unsuccessful, because the frog tissue often failed to cover the endodermal plug, although at the time of grafting the tissue seemed ample for the wound bed. A few isolated cases, however, from the Rana pipiens material may be worth recording for in certain particulars they reveal responses on the part of the host very similar to those occurring after homoplastic grafts.

Ten experiments were performed in which Rana pipiens was the donor. In these cases stomodaeal ectoderm and occasionally a few mesodermal and endodermal cells composed the graft. Five specimens lived to stages 43–46, so it was possible to see whether a mouth opening was formed. In one case no opening was to be found and the condition was in every respect similar to that of a 'no contact' specimen in the homoplastic series or even in the regeneration series. The meckelian cartilages were anterior to the blind end of the foregut and the massed splenial and palatine teeth appeared dorsal to the hyomandibular cleft.

The particular interest in this specimen, however, was the presence of a 'maxillary bulb' far anterior in the head region. This was plainly the derivative of Amblystoma tissue, and at once suggested that this ectoderm possesses some specificity for tooth formation if left in its normal location. One is again led to believe that the ectodermal epithelium must take the initial step in tooth formation, but absolute proof is lacking here as in the cases of 'maxillary bulbs' already recorded for the previous experimental series. No hypophysis was discovered in this specimen and the melanophores were markedly contracted.

In another case (stage 46) a minute opening less than 10 μ in width was found. At stage 34 the frog tissue completely covered the stomodaeal region. At stage 36, according to the record, a small indentation was evident in the middle of the graft, while at stage 43 a small white projection and a minute pit could be seen there. The external appearance at stage 46 suggested the possible presence of an opening in the projecting area and examination of sections shows that a small hole is present. The frog graft is connected with the endodermal plug over a limited area, but the small cells of this graft have formed a solid plug instead of an ectodermal collar. The opening which connects with this plug is so minute that it is not convincing evidence for the formation of a well-developed oral opening through overlying frog tissue.

In the three remaining specimens there were mouth openings which corresponded with the portion of the endodermal plug which had been left uncovered by the graft, and conversely the arrangement of the mouth structures agreed with the degree of adaptation necessitated by the presence of the frog tissue. For example, in two specimens oral openings and well-formed lip folds were found on the left sides which were not covered by the grafts. In another specimen a minute opening was present in the midline where a very small endodermal plug had remained uncovered.

As in the transplantation series, so in this, the arrangement of meckelian cartilages may be interpreted according to the extent of the barrier which had existed in the plug contact whether covered or not by grafted tissue. When the opening was on the left side, the foregut was shifted to that side, and the right meckelian cartilage was displaced medianly and more internally than the left. If a small median contact was present, the cartilages formed a sharp V around the small foregut. When no contact existed, the meckelian cartilages were located in front of the blind end of the foregut just ventral to the brain.

These specimens are too few in number to present conclusive evidence as to the effect of heteroplastic grafts. However, the results thoroughly agree with those of the experiments with homoplastic grafts. Thus, the relation of the teeth, the correlation of the mouth opening with the size of the plug, the arrangement of the meckelian cartilages, and even the formation of 'maxillary bulbs' coincide with the previous findings. They further suggest that similar demands call forth similar responses on the part of the individuals concerned and also confirm the view that the relation of the foregut to the ectoderm of the oral plate, whether this ectoderm is normal, regenerated, homoplastic, or heteroplastic tissue, is of paramount importance in the development of the mouth.

4. Transplantation of homoplastic stomodaeal ectoderm to the gill region (GSt series). In addition to the regenera-

tion and transplantation experiments already recorded, another series of operations, the reciprocal of the gill-transplantation group, was made to test the power of self-differentiation of the stomodaeal ectoderm. In this experiment the stomodaeal ectoderm (cut 1+) was placed on the gill region after the gill ectoderm had been removed. A few mesodermal cells were lost in the removal of the gill ectoderm, but this loss was generally so slight that it was probably not sufficient to invalidate the experiment as far as the stomodaeal ectoderm is concerned. The embryos used ranged from stage 23–27, but the complete agreement regarding tooth formation indicated that the slight age differences included within these limits caused no variation in the results. For two or three days after the operation it was often possible to identify the puffy contour of the ectoderm normally located over the arches in strong contrast to the thin ectoderm of the oral plate. The graft was oriented in various ways, and in every case there was a correlation between the orientation and the structures which developed from the graft.

The external appearance of these animals presented certain features which agreed with the findings of Ekman ('13) on Anura and Harrison ('21) on Urodeles, concerning the suppression of the development of gills by grafts from another region of the body. The gills on the operated side were always imperfect. Sometimes the defect involved portions of all three of the gills, although in no case were the three wholly suppressed. In other cases only two or parts of two gills were affected. In certain instances it was evident that the gill filaments had arisen from the normal gill anlage, because no stained tissue appeared on the branches. In other specimens, however, the filaments were covered with stained ectoderm, or in still others the region from which a gill would normally arise showed no indication of sprouts, the territory being covered wholly with the grafted tissue. These data support Harrison's conclusion that incomplete suppression results when ectoderm is grafted from a region of the head fairly near the normal position of the gills. In several cases

a balancer was present on the part of the graft, and its longitudinal axis was always in accord with the orientation of the graft. Often one, or rarely two, transparent spots with dark centers appeared within the limits of the grafted ectoderm which an examination of sectioned material proved to be lenses and confirmed the findings of Harrison ('20) on the independent differentiation of the Amblystoma lens after transplantation.

The particular significance of this experiment, however, concerns the possibility of the formation of teeth from the grafted tissue. No tooth germs were found in any instance, although the ectoderm had invaginated slightly in certain spots in addition to the ingrowths which had given rise to the nasal sac and lens structures (fig. 71) in specimens ranging from stage 39 to stage 45. Three individuals in stages of development younger than 39 were also carefully examined for any trace of dental papillae on the assumption that a germ might start to develop in an early stage and then be resorbed. No evidence for this occurrence was discovered.

The absence of tooth germs in connection with the graft is the more striking in that it is obvious that the gill region presents more favorable conditions for the development of teeth than any in the body except the normal position. The particular advantages of the region are the nearness of the endoderm to the surface in the gill pouches and the presence of mesectoderm, which superficially seems similar to the mesectoderm normally giving rise to the papillae of the teeth. From this failure to form tooth germs in addition to the fact that they may apparently be developed from ectoderm transferred to the stomodaeal region from another part of the body, as seen in the previous experiments, the inference may be drawn that the development of teeth in Amblystoma is limited to an area in the ventral part of the head which in early stages is located between the end of the forebrain and the heart anlage. Furthermore, the development appears to be restricted to the ectoderm which invaginates over the endoderm except in cases of the 'maxillary bulbs,' or to the

endoderm posterior to the collar, and it is probable that the epithelium of these layers is responsible for the initiation of the development of the tooth germ. However, it also seems probable that the mesectoderm from the neural crest of the trigeminal region possesses a distinct specificity for the particular interaction with the ectoderm and endoderm which results in a fully formed tooth germ.

DISCUSSION

The study of the normal development of the mouth of Amblystoma punctatum and the results of the experiments recorded in this paper present evidence on three points in particular. First, it seems fair to consider the development of the anterior part of the oral cavity in this species as a modification of the usual formation of the vertebrate stomodaeum by a bay-like invagination of ectoderm. Secondly, the derivation of the enamel organs of the teeth in this form is not limited to the ectodermal germ layer which invaginates around the solid endodermal column of the foregut. Thirdly, there is a sequence of steps in the formation of the mouth which is of great importance in the developmental mechanics of the head.

Technically, the vertebrate stomodaeum is that part of the anterior end of the alimentary canal which is formed by an ingrowth of ectoderm and therefore possesses an ectodermal lining as does the proctodaeum at the posterior end of the alimentary canal. Is there any part in the anterior region of the alimentary canal of Urodeles which answers this description and which, therefore, may be termed a stomodaeum? Conflicting views are to be found in the researches of Johnston, Landacre, Kingsley and Thyng, and Kerr, which bear on this point, as has been shown in the introduction. Can these conflicting views be reconciled?

The study of normal stages from 34–46 has made it seem more than probable that the idea held by Kerr is erroneous, and that an invagination of ectodermal tissue does occur and forms the 'ectodermal collar' of Johnston and Landacre.

This opinion is based on a comparison of the size of the collar at various stages of growth, the presence and arrangement of mitotic figures within its limits, and the lack of any indication that endodermal cells are merely rearranging themselves in a columnar epithelium and through their great metabolic activity in digesting the large yolk granules, are taking on the appearance of ectodermal cells. The evidence from the normal stages, however, is indirect, but that from experiments is more convincing.

In the series of operations in which the stomodaeal ectoderm is removed but not replaced by other tissue, the subsequent formation of the collar is noticeably delayed. The developmental processes in the region normally just posterior to the internal collar boundary, however, continue at an apparently normal rate. This is evidenced by the presence of a thin collar with flattened cells abutting against a thickened endodermal region in which developing tooth germs may be seen. If the collar is a transformation of endoderm in situ, there is no plausible explanation of the retarded condition, because in this operation only the ectoderm is removed and the endoderm is left intact to continue its normal development. Nor is any collar formed by the transformation of endodermal cells into an apparently ectodermal structure in the cases in which no contact between ectoderm and endoderm is established, although again the endodermal cells have not been removed.

Since the evidence indicates that an invagination occurs to form an ectodermal collar, may it be considered a modification of the usual stomodaeal formation or does the fact that it is separated from the cavity of the mouth by endoderm technically preclude this possibility? Kingsley and Thyng say that the endodermal cells within the ectodermal collar disappear completely, but I am inclined to doubt this observation and to agree with Johnston and Landacre that the endoderm really separates the ectodermal collar from the cavity of the mouth. I have observed cells with large yolk granules in stages 45–46 even at the edge of the oral opening and

there appears to be no indication of a disintegration of this seemingly endodermal layer. However, it may disappear later, and if so the technical difficulty of an endodermal lining in the region which these authors call a modified stomodaeum would not need consideration.

But even if the endoderm does persist as the lining of the part of the oral cavity encircled by the ectodermal collar, are we to agree with Johnston that no stomodaeum is formed although an ectodermal invagination has occurred? Or may we take the middle course with Landacre that the region bounded by the collar is homologous with the stomodaeum of other vertebrates? I am inclined to agree with the latter view not only because the collar seems to be a developmental adjustment to the peculiar thickness of the endodermal component of the oral plate in Urodeles, but also because this region possesses the derivatives, particularly the maxillary and dentary tooth germs, and a relation to other structures of the head similar to those in the stomodaeal region of other vertebrates. Furthermore, the experimental data as to the method of ingrowth and the abnormalities following any failure in its formation seem to confirm this belief.

The evidence that only a certain proportion of the enamel organs of the tooth germs of Amblystoma punctatum is formed from the invaginated ectodermal collar is irrefutable. This has been demonstrated in the normal stages ranging from 37–46, in which the earliest tooth anlagen (splenial) exist in the endoderm of the lower jaw and in later stages many tooth germs are found in this position and in the endoderm of the palatine region. But the most convincing evidence of endodermal enamel organs exists in the results of those experiments in which no contact is established between ectoderm and endoderm to form an oral plate. This condition may occur either in the regeneration series or in the homoplastic or heteroplastic transplantation series. In these, the only tooth germs present are lodged in the endodermal tissue and can be assigned to the palatine and splenial series. Conversely, the tooth germs normally located in the ectodermal collar are missing because of the absence of this structure.

The existence of teeth similar in structure, but with enamel organs derived on the one hand from ectoderm (maxillary, vomerine, dentary) and on the other hand from endoderm (palatine and splenial), raises the question of the specificity of germ layers. Largely on the basis of the extensive researches of O. Hertwig, the placoid scale found in the integument of Elasmobranchs which unquestionably possesses an ectodermal enamel organ is considered the homologue of the vertebrate tooth.[4] Moreover, the invagination of ectoderm, really embryonic integument, which forms the stomodaeum and the derivation of teeth from this invaginated tissue, bear out this contention. Therefore, the opinion prevails that the presence of teeth in any region is an accurate criterion for the existence of the ectodermal germ layer in such region at some time in development. But, as we have seen in the introduction, some investigators are disturbed by the presence of tooth germs posterior to the oral plate in a region which is certainly lined with endoderm—a condition which occurs among some of the Elasmobranchs, Teleosts, and Amphibia. A few of these therefore advanced the suggestion that after the perforation of the oral plate, the ectoderm migrated caudally over this region, replaced the endoderm, and gave rise to the enamel organs of such teeth as were here present. Cook and Neal, however, from a critical study of successive stages of Squalus embryos, discover no traces of such caudal migration of ectoderm after the perforation of the oral plate. They, therefore, agree with others who definitely state that they believe the enamel organs present on the pharyngeal teeth of Teleosts, on the palatine teeth of Urodeles, and on the pharyngeal scales of Elasmobranchs are derived from the endoderm. On the other hand, we can scarcely take the extreme stand which Kerr takes in saying that all of the teeth in Urodeles have endodermal enamel organs, for the lack of ectodermal tooth derivatives in 'no contact' cases demonstrates the inaccuracy of such a statement.

[4] Owen in 1845 discussed this homology at some length, but he did not concern himself with the derivation of the parts from particular germ layers.

These opinions, together with the data presented in this paper, certainly offer strong evidence against the strict specificity of the germ layers in the formation of the enamel organs. Similarity does not prove that structures originate from the same germ layer in every case. As is well stated by Cook and Neal, "the force of the doctrine of the specificity of the germ layers has been greatly weakened by abundant evidence of the plasticity of regenerating tissue," and these authors cite cases of budding in colonial Tunicates and regeneration processes in Chordates in support of their view. To these might be added similar instances in Arthropods and Annelids and probably in Echinoderms, where in regeneration, muscle and connective tissue may be formed from ectodermal epithelium.

In addition to the problem of the derivation of the enamel organs from ectoderm or endoderm, the question arises as to the sequence of events in the formation of a single tooth germ and the rôle which the germ layers, that is, ectoderm and mesectoderm, or, endoderm and mesectoderm, play in this formation. Röse repeatedly states in his published works that the epithelium takes the initial step in the formation of the tooth germ, and his opinion is upheld by many later investigators. The irritation caused by the thickening in the epithelium serves as the stimulus to the underlying mesoderm to grow more actively and to press into the cylindrical cells of the epithelium like a cone. Moreover, the shape of the tooth and the area over which the dentine will be secreted are said to be dependent on the epithelial layer. On the other hand, one occasionally finds a description of a placoid tooth which assigns to the mesoderm of the papilla the initial impetus in development.

Röse's view as to the importance of the epithelium seems confirmed in these experiments by the abnormal arrangement of the palatine and splenial teeth when the oral plate is lacking. This corresponds to the abnormal massing of the endoderm. However, this appearance does not rule out the possibility that the mesectoderm plays more than a passive

part in development. 'Maxillary bulbs' seem to be the direct result of a thickening of the ectoderm. But, as in the other case, mesectoderm certainly participates in these tooth germs, and in both cases it is for the present impossible to state which of the germ layers takes the lead in initiating development. It is certainly not probable that a tooth would develop far without the specific mesectoderm composing the pulp on which the production of dentine depends—a point which seems to gain support from the lack of self-differentiating potentiality on the part of the stomodaeal ectoderm when transplanted to the gill region. Either the specificity of this ectoderm for giving rise to enamel organs is not established at the time the ectoderm is removed (stages 23–27) or the foreign position and the absence of the tissues which normally interact with it, namely, the mesectoderm and the endoderm of the oral region, result in the inhibition of the development of tooth germs. This non-development is particularly striking because supernumerary lenses, nasal sacs, and balancers form from the grafted tissue.

That failure of mesectoderm and probably endoderm to react with ectoderm is the correct diagnosis of the lack of tooth germs in stomodaeal transplants to the gill region is further supported by those cases in which the oral plate is not formed. In the 'contact' cases of this series, that is, when an oral plate is present, the regenerated ectoderm gives rise to maxillary, vomerine, and dentary tooth germs as the derivatives of an invaginated collar. In 'no contact' cases, however, although regenerated ectoderm is present over the oral region, its failure to invaginate around an endodermal column leaves it in an abnormal position and no dental papillae are formed except in the rare cases of 'maxillary bulbs.' Furthermore, the probability of interaction is supported by the formation of enamel organs from ectoderm belonging to a foreign graft. Therefore, 'no contact' cases and these just cited imply that the stomodaeal ectoderm of Amblystoma punctatum is not self-differentiating, but is dependent on a certain relation with the endoderm before the formation of

teeth will normally begin. After that relationship is established the question of the primary activity on the part of the ectoderm or mesectoderm still remains unsettled, as in the case of the endoderm and the mesectoderm, and the decision as to the rôles of the respective germ layers in the formation of a tooth must rest on further experiments.

Another question which is raised by the results of the foregoing experiments concerns the factors involved in the formation of the mouth. The sequence of events in the normal process includes the establishment of the one-layered ectoderm, the formation of the ectodermal collar, and finally the perforation of the oral plate. This is not only a time sequence, but it is also a chain of events in which each step is dependent on the preceding one. The initial step, the establishment of the one-layered ectoderm in the oral plate, is the result of the withdrawal of the inner layer of ectoderm due to an interaction with the endoderm. This withdrawal also occurs when grafted ectoderm is present in contact with the endoderm, and it fails to occur when no ectodermal-endodermal contact is established whether foreign or regenerated ectoderm is present over the oral region.

The far-reaching effects of the early contact are evidenced by the size of the collar and the size and position of the oral opening in later stages. This is repeatedly substantiated by an examination of the records of individual specimens. If a plug was present either on the left or on the right side, or in the median line in an animal of the regeneration series, and if the specimen was kept until a stage when a mouth opening should be formed, a perforation was found on the left or the right side or in the median line in accordance with the previously recorded position of the plug. Also if a plug showed through the grafted ectoderm in the transplantation series, it was prophetic of a future oral opening similar in size and location to the plug. Thus it is evident that the shape and size of the mouth are largely determined in stages 25–29, when the one-layered condition of the ectoderm is being established.

The second step in the process of the formation concerns the development of the collar. The ectoderm normally invaginates around the endodermal column of the foregut which is attached to the ventral ectodermal area just between the forebrain and the heart. Thus if the shape of the foregut varies through imperfect attachment to the ectoderm, it would be expected that the collar would also be imperfect. Accordingly, if a small contact exists, a small collar wraps itself around the reduced end of the foregut, and if the foregut possesses an attachment to the ectoderm on only one side, the anterior part of the foregut, including the collar, will be located lateral to the median line of the animal.

It must be noted also that abnormalities in the meckelian cartilages and to some extent in the cartilages of the other visceral arches are due primarily to the imperfection of the oral plate. This is most conspicuous in those cases in which the oral plate is completely lacking, for here the mesoderm and mesectoderm have had access to a region normally barred to them by the presence of the endoderm of the foregut. With this in mind, it is not surprising to find large and often irregular meckelian cartilages forming a wall in front of or even somewhat dorsal to the blind end of the foregut. In other cases in which a contact has occurred at one side of the median line, the meckelian cartilage of the other side is lodged further dorsally and medianly than normal in the space usually occupied by half of the foregut tissue, whereas on the normal side the cartilage is found in its regular position. In lesser degree, the branchial cartilages and the hyoid arch exhibit some modification in position, rarely in shape, due to the abnormal cramping of the tissue behind the mouth and the failure to develop the normal shape of the head.

From these illustrations an idea may be gained of the importance of the oral plate and of the endodermal column extending posteriorly from this union for the developmental mechanics of the head. The column serves as a fixed axis, attached at the oral plate, on which the visceral arches and particularly the mandibular arches and their maxillary proc-

esses are molded. While there are unquestionably other factors involved in shaping the head, such as straightening of the flexures, enlargement of regions due to unequal growth, and shifting of parts, the arrangement of the finished structure is certainly dependent to a large degree on the early condition of the anterior end of the foregut and the oral plate.

SUMMARY

1. Stages in normal development and in operated animals demonstrate the presence of endodermal as well as ectodermal enamel organs in the tooth germs of Amblystoma punctatum. Occasionally an enamel organ may be composed partly of ectodermal and partly of endodermal cells, as is true in the median pair of dentaries. The teeth which possess endodermal enamel organs belong to the palatine and splenial series, while the maxillary, vomerine, and dentary groups, with the exception already noted, arise wholly within the ectodermal collar.

2. The appearance of tooth germs in Amblystoma occurs in the following sequence: splenial, vomeropalatine, dentary, and maxillary. The earliest germs found, namely, the splenials, are of the 'placoid type,' whereas the last to form develop from a dental ledge.

3. The evidence from normal stages and particularly from operated animals indicates that the ectodermal collar is a true invagination and not a transformation in situ of endodermal cells. Moreover, the formation of the ectodermal collar may be considered a modification of the usual process of stomodaeal formation in vertebrates.

4. The formation of an ectodermal collar is essential for the development of maxillary, vomerine, and dentary teeth and also for the establishment of the oronasal connections.

5. There is a definite interaction between the ectoderm and endoderm of the oral plate which brings about the one-layered condition of the ectoderm. When contact between these two germ layers does not occur, the two-layered condition of the ectoderm persists and a collar and oral opening are not formed.

6. The most important 'moment' in the formation of the mouth is the period of the establishment of the one-layered ectoderm. The degree of normality subsequently attained is dependent on the degree of perfection of this early contact between the endoderm and the ectoderm. Thus, there is a definite correlation between the size and the position of the contact in early stages and the oral opening found in later stages.

7. No tooth germs form in stomodaeal ectoderm transplanted to the gill region. However, foreign ectoderm transplanted to the stomodaeal region may apparently develop tooth germs when associated with the tissues of this region.

8. The removal of a small amount of stomodaeal ectoderm is followed by more complete and perfect regeneration of this tissue than is the case when a large amount is removed. This is seen in the development of the approximately normal shape of the mouth and ectodermal tooth germs in many of the regeneration series in which a small cut was used. All of the specimens of this series show that the stomodaeal ectoderm possesses a marked regenerative capacity.

9. The stage at which the operation of removing stomodaeal ectoderm is performed probably has some influence on the results of the operation. The experiments suggest that either an early stage, 19–23, or at a late one, 27–30, is preferable to an intermediate one, 24–25. This may be due to the fact that during this intermediate period the visceral arches are in active development, while earlier they are just beginning to show signs of activity, and later their pattern has become well formed.

10. Complete absence of the hypophysis due to the removal of its anlage with the stomodaeal ectoderm noticeably influences the color of embryos in stages 42–46. Such animals may be distinguished from normal ones by the marked contraction of their melanophores. Mention of 'paleness' in the records is correlated with lack of hypophysial cells when sectioned material is examined.

11. There is in the ectoderm of the ventral body wall and that of the gill region a difference in growth potency. That of the latter is much greater than that of the former, as may be seen in the subsequent development of the grafted tissue in similar operations.

12. The results of the experiments indicate that the visceral arches, particularly the mandibular pair with its maxillary processes, are molded around the fixed but plastic axis of the foregut, and that they depend on the proper placing of this endodermal tissue for their normal developmental relations.

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PLATES

ABBREVIATIONS

AEC, anterior component of ectodermal collar
AT, anterior trabecula
AV, auditory vesicle
B, brain
BAL, balancer
C, cerebrum
CL, cerebellum
D, dentary tooth germ
DI, dienchephalon
E, eye
ECT. 1, one-layered ectoderm of oral plate
ECT. 2 two-layered ectoderm over mouth region
FG, foregut
H, hypophysis
HC, hyoid cartilage
HH, hyohyal
INF, infundibulum
L, lens
LE, lateral extension of mouth cavity
M, maxillary tooth germ
"MB" 'maxillary bulb'
MC, Meckel's cartilage
MED, medulla
MES, mesencephalon
MS, mesoderm
MTH, mouth
MY, muscle
N, nasal sac
NC, nerve cord
NCH, notochord
ONP, projection for oronasal connection
OP, oral plate
OPC, oropharyngeal cavity
P, palatine tooth germ
PCH, parachordal cartilage
PEC, posterior component of ectodermal collar
POP, posterior edge of oral plate
PQ, palatoquadrate bar
S, splenial tooth germ
T, tongue
V, vomerine tooth germ
VN, vomeronasal organ
VP, vertebral process
1–2 BB, first and second basibranchial cartilages
1–4 BC, first to fourth branchial cartilages
II, optic nerve

PLATE 1

EXPLANATION OF FIGURES

Throughout these figures the ectoderm is shown by stippling, the endoderm by small rings. Mesoderm is omitted from the figures except in the case of cartilages.

1 to 8 Ventral views of embryos of Amblystoma punctatum, showing shape of oral plate in stages 28, 34, 35, 37, 38, 40, 41, and of open mouth in stage 43. × 10.

9 to 11 Sagittal sections of an embryo, stage 35 (fig. 3), showing ectodermal collar. × 33.

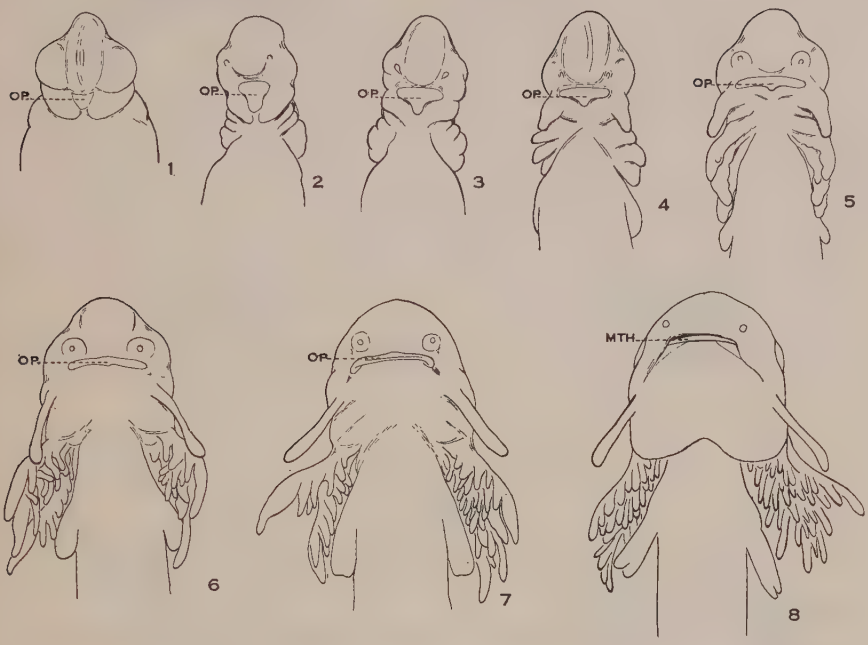
9 Section through edge of oral plate, showing arch of ectodermal collar.

10 Section nearer the median line, showing separate anterior and posterior components of the collar.

11 Section approximately through median plane of foregut, showing hypophysis (heavily stippled) and absence of collar invagination at posterior median edge of oral plate.

12 to 21 Diagrams showing extent of ectodermal collar and endodermal tissue and location of tooth germs as viewed from outer surface of foregut in stages 37, 39, 41, 42, and 45 of Amblystoma punctatum. The key to the symbols is as follows: Stippling, :::, ectodermal collar; heavy dots •••, posterior edge of ectodermal collar; small circles, ○○○, endodermal tissue; large circle, ○, tooth germ—relative development of tooth germs shown by thickness of rings; outer and inner solid lines, ——, contours of head and foregut, respectively; dashes, – – –, outline of cavity of foregut; dots and dashes –·–·–, anterior edge of corresponding dorsal or ventral collar in ventral or dorsal view of foregut, respectively. Description of diagrams given in text. × 33.

12 and 13 Roof of mouth (dorsal) and floor of mouth (ventral), respectively, stage 37.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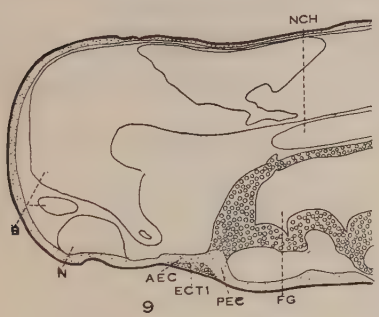
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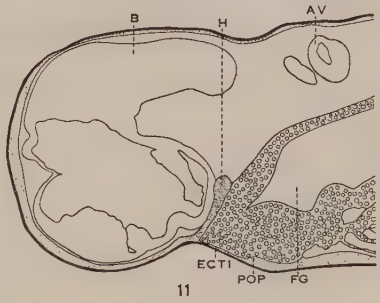
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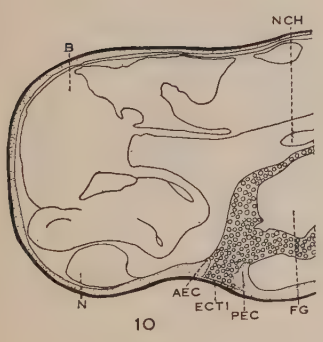
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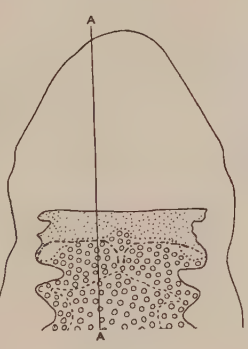
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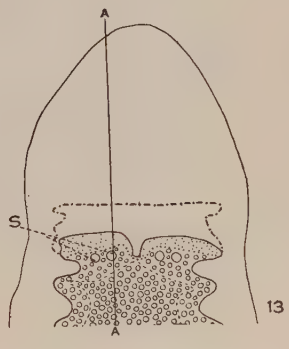
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PLATE 2

EXPLANATION OF FIGURES

12 to 21, continued.

14 and 15 Roof of mouth (dorsal) and floor of mouth (ventral), respectively, stage 39.

16 and 17 Roof of mouth (dorsal) and floor of mouth (ventral), respectively, stage 41.

18 and 19 Roof of mouth (dorsal) and floor of mouth (ventral), respectively, stage 42.

20 and 21 Roof of mouth (dorsal) and floor of mouth (ventral), respectively, stage 45.

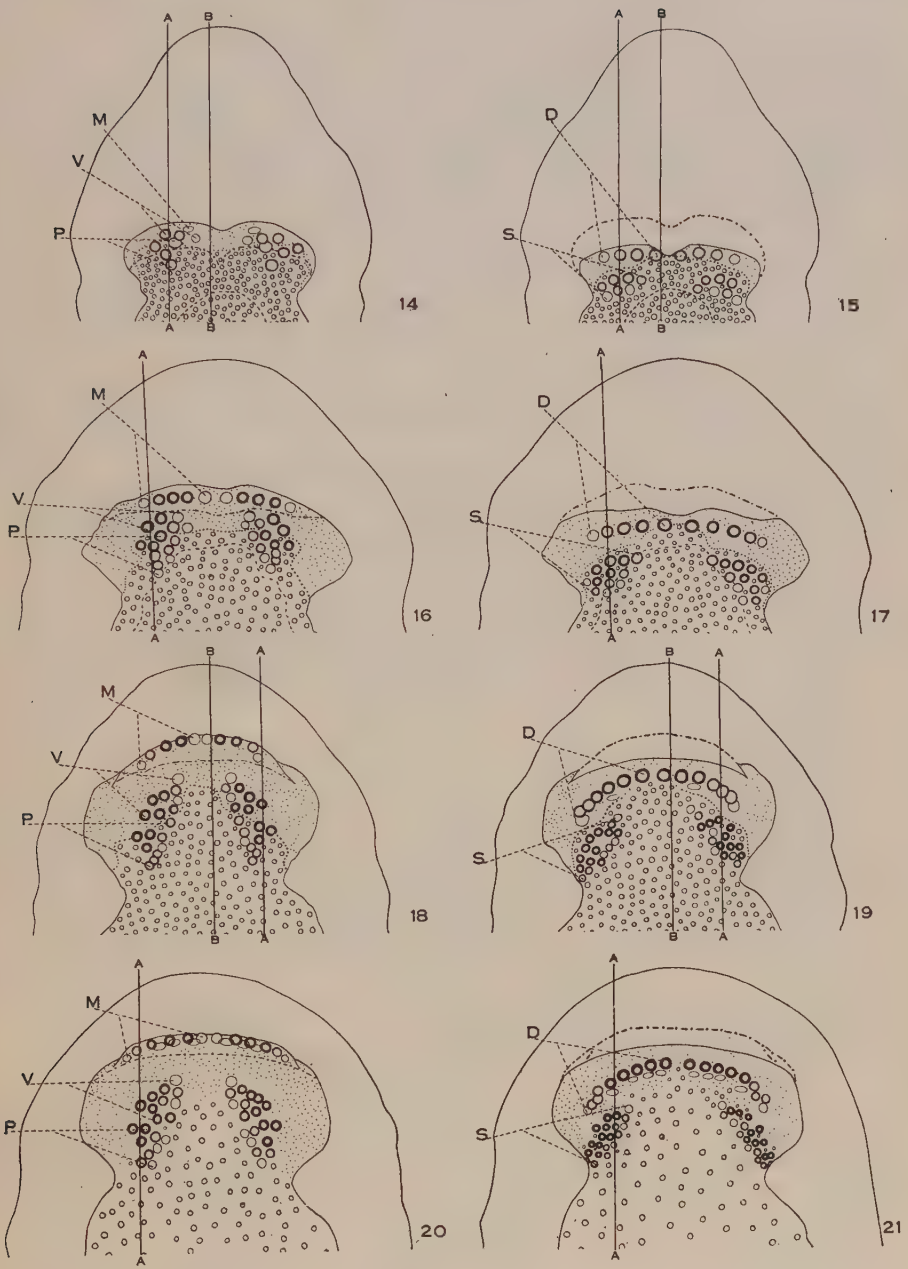
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PLATE 3

EXPLANATION OF FIGURES

22 Sagittal section through embryo, stage 37, along line A–A in figures 12 and 13, showing collar and splenial tooth germ. × 33.

23 Sagittal section, stage 39, through line A–A in figures 14 and 15, cutting one vomerine, two palatine, one dentary, and two splenial tooth germs. × 33.

24 Sagittal section, stage 39, through line B–B in figures 14 and 15. One of the median dentary tooth germs with an ectodermal-endodermal organ is cut in this section. × 33.

25 Sagittal section, stage 41, through line A–A in figures 16 and 17, in which parts of one maxillary, one vomerine, two palatine, one dentary, and four splenial tooth germs are cut. × 33.

26 Sagittal section, stage 42, through line A–A in figures 18 and 19, in which one maxillary, one vomerine, two palatine, one dentary, and four splenial tooth germs are cut. × 33.

27 Sagittal section, stage 42, through line B–B in figures 18 and 19, in which the maxillary and dentary ledges are cut. × 33.

28 Sagittal section, stage 45, through line A–A in figures 20 and 21, in which parts of one maxillary, one vomerine, two palatine, one dentary, and three splenial tooth germs appear. × 33.

29 Ventral view of embryo, stage 24, showing extent of cuts used in operation. The small rectangle bounded by small dashes represents cut 1; the larger rectangle bounded by large dashes shows cut 1+; cut 2 extends over an area equal to that of 1+ and an additional anterior section outlined by the dash-and-dot line. × 10.

30 Median sagittal section of embryo, stage 34, after removal of stomodaeal ectoderm by cut 1+. Mesoderm separates the blunt end of the foregut from the two-layered ectoderm over the mouth region. × 33.

31 Sagittal section of embryo St21b, stage 39, showing delayed formation of ectodermal collar, while endoderm has given rise to splenial and palatine tooth germs. One layer of ectoderm covers the endodermal plug. × 33.

32 Sagittal section through the lateral part of the ectodermal collar of a specimen, stage 37, showing inequality of development in anterior and posterior components of the collar following removal of stomodaeal ectoderm. × 33.

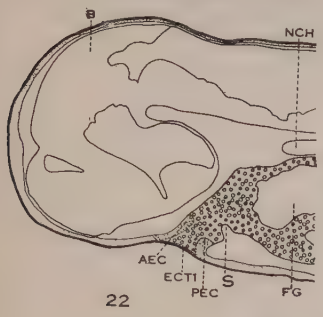
33, 34, 35 Sagittal sections through collar of specimen St20a, stage 38, showing irregularity in development of the anterior component of the collar following removal of stomodaeal ectoderm. × 33.

33 Thickened collar, bulb-like invagination of tissue, right side.

34 Indentation of collar by a clump of mesectoderm cells, right side.

35 Normal collar shape, left side.

36 Sagittal section of specimen St37a, stage 40, showing well-formed ectodermal collar with one maxillary, one vomerine, and one dentary tooth germ within its limits and one layer of ectoderm covering the endodermal plug. The stomodaeal ectoderm was removed by cut 2, stage 27–30. × 33.



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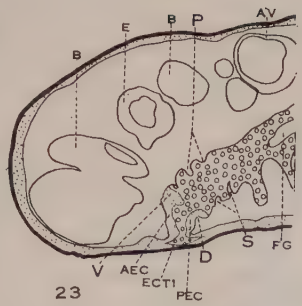

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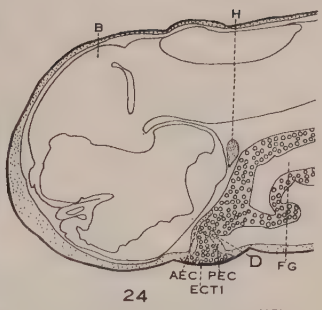

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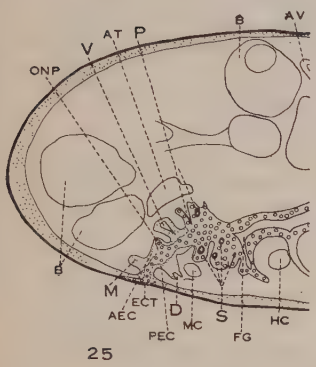

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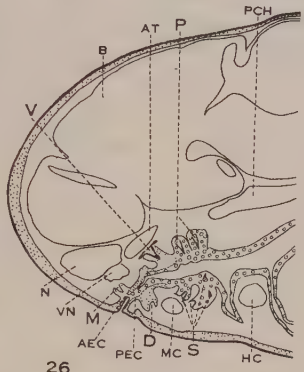

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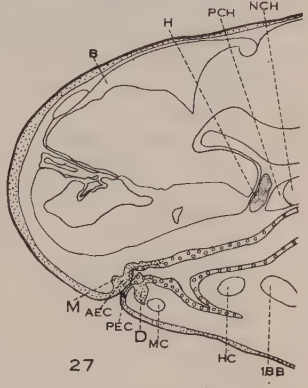

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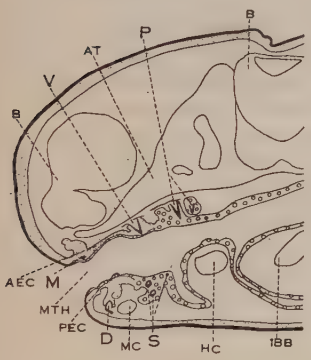

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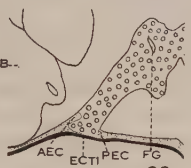

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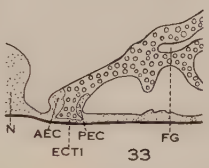

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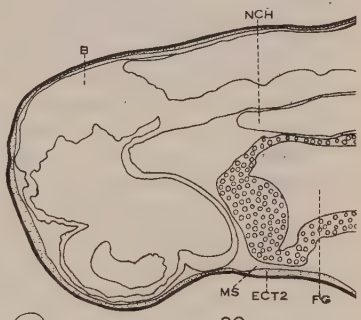

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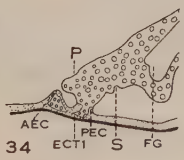

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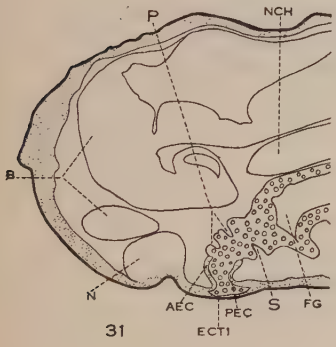

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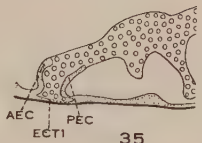

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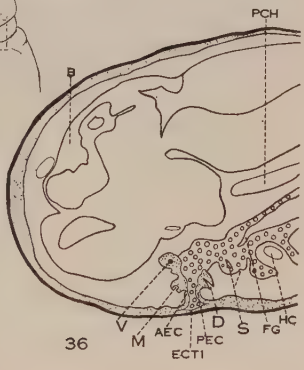

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PLATE 4

EXPLANATION OF FIGURES

37 Median sagittal section of specimen St45d, stage 40, showing lack of contact between endoderm of gut and two-layered ectoderm. Three tooth germs of splenial (possibly of palatine) tooth set are present at the blind end of the foregut. Gut endoderm projects between notochord and brain. The brain is abnormally cramped and shows enlarged sac-like infundibular region. × 33.

38 Transverse section of specimen, stage 45, showing well-formed lateral extension of the mouth and separation of palatine and splenial tooth germs on the left side (right of figure), while on right side (left of figure) the lateral extension is nearly lacking, splenial and palatine tooth germs are approximately in contact, and the meckelian cartilage is abnormally situated. This specimen had its mouth opening on the left side (corresponding to right of figure). × 33.

39 Sagittal section of specimen St45e, stage 45, showing a 'maxillary bulb' separated from any connection with the foregut. Two layers of ectoderm cover the mouth region, since there is no contact of ectoderm and endoderm. One palatine tooth is cut in the section. × 33.

40 Transverse section of specimen St27c, stage 46, showing location of 'maxillary bulb' between nasal sacs. × 33.

41 Transverse section of specimen St27c, fifty-one sections posterior to section in figure 40. The small tubular foregut, bases of two dentary tooth germs, and three sections of the meckelian cartilages in an abnormal relation to the foregut tissue may be seen. In region marked x it is uncertain whether cells are ectodermal or endodermal in origin, but probably they are ectodermal. × 33.

42 Transverse section of specimen St27c, seventeenth section posterior to section in figure 41, showing narrow mouth cavity and abnormal arrangement of splenial and palatine tooth germs and meckelian cartilages. × 33.

43 to 46 Outlines of heads of four specimens, stages 44–45, group StI, operated in stages 19–23, cut 1+, showing variations in shape and position of mouth. × 10.

43 Mouth consists of two small perforations in median line.

44 Shape of mouth and development of lip folds approximately normal.

45 Small mouth on left side of head.

46 Small mouth on right side of head; mandibular-hyoid region slightly protruding.

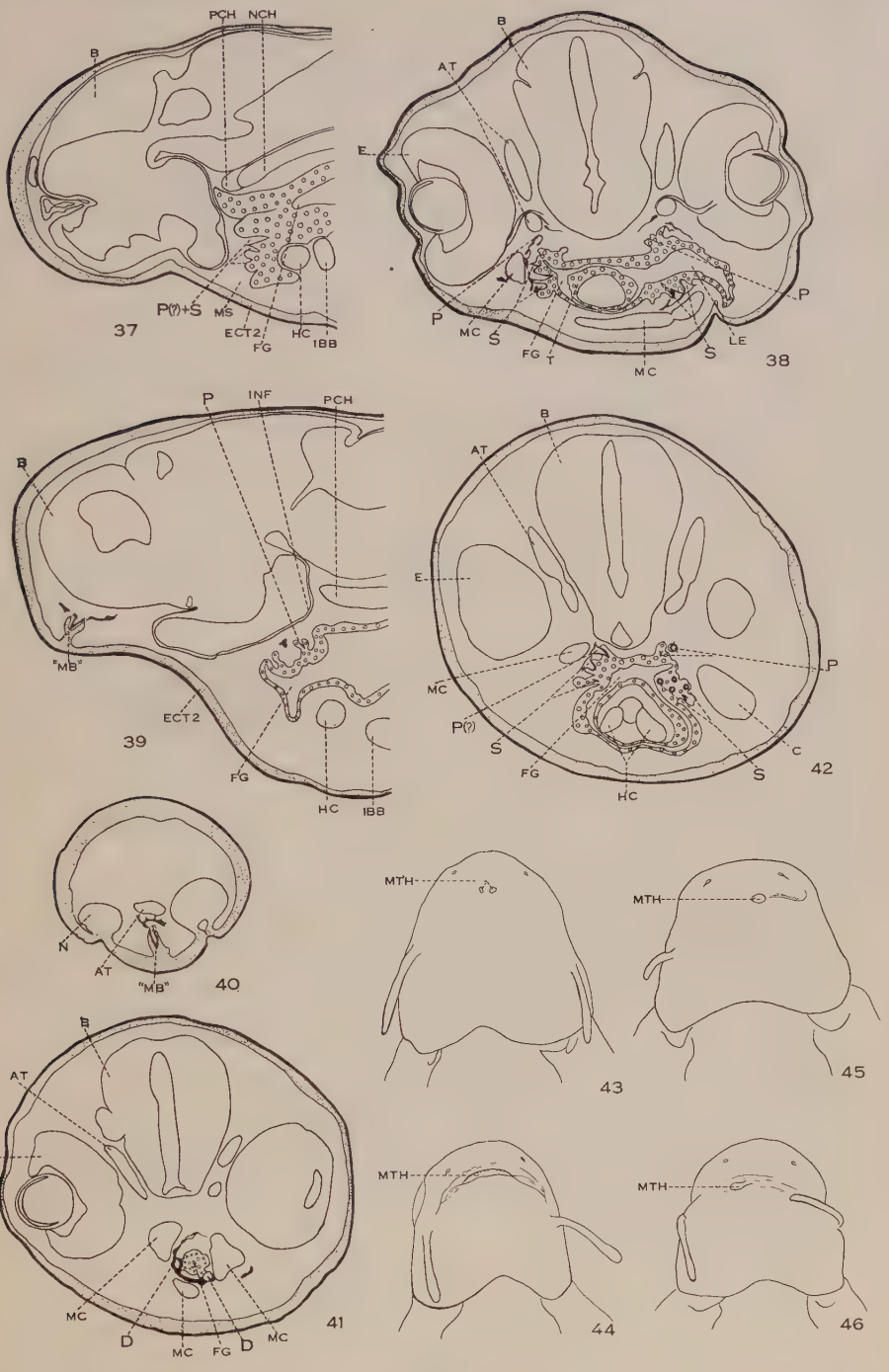
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PLATE 5

EXPLANATION OF FIGURES

47 to 51 Outlines of heads of four specimens, stages 43–45, group StII, operated in stages 24–25, cut 1+, showing variation in shape and position of mouth. × 10.

47 Small mouth opening in median line.

48 Very small mouth opening in median line.

49 Small mouth opening on right side.

50 No mouth opening present.

51 Lateral view of specimen of figure 50, showing protruding branchial region of a 'no contact' animal.

52 to 53 Outlines of head of specimen belonging to group StIII (operated stages 27–30, cut 1+), to show moderately normal mouth formation in this group. × 10.

52 Ventral view of specimen.

53 Lateral view of specimen. Lower jaw region is slightly reduced.

54 to 55 Left lateral views of specimen StG2 and its normal control, stage 36, showing flattened mandibular arch region and abnormal flexure of head in operated specimen. × 10.

54 Control specimen.

55 Operated specimen; grafted tissue is outlined by dotted line.

56 Sagittal section of specimen StG13, stage 39, cutting collar of grafted tissue. Tooth germs are present in other sections of the collar. One layer of ectoderm covers the endodermal plug of the foregut. × 33.

57 Sagittal section of specimen StG107, stage 38, showing well-formed collar containing one vomerine tooth germ. The collar is apparently formed from grafted gill ectoderm and one layer of ectoderm covers the endodermal plug. A splenial tooth germ may be seen in the endoderm. × 33.

58 Sagittal section of the same specimen, StG107, stage 38, showing two palatine and two splenial tooth germs in the endoderm. × 33.

59 Sagittal section of specimen StG52, stage 45, passing through the small contact area of this animal. A minute perforation opens into the gut cavity in this specimen. In region marked x it is uncertain whether cells are of ectodermal or of endodermal origin, but it seems probable that an ectodermal collar has formed, although it contains no tooth germs. Parts of five splenial tooth germs are cut in this section. × 33.

60 Sagittal section of specimen StG116, stage 42, cutting an ectodermal tooth germ in a 'maxillary bulb' and one palatine and three splenial tooth germs in the endoderm. The 'maxillary bulb' appears to be derived from grafted gill ectoderm. The oropharyngeal cavity is represented by small cavities in the gut tissue. The region marked x probably belongs to ectodermal collar, but its extent is uncertain. × 33.

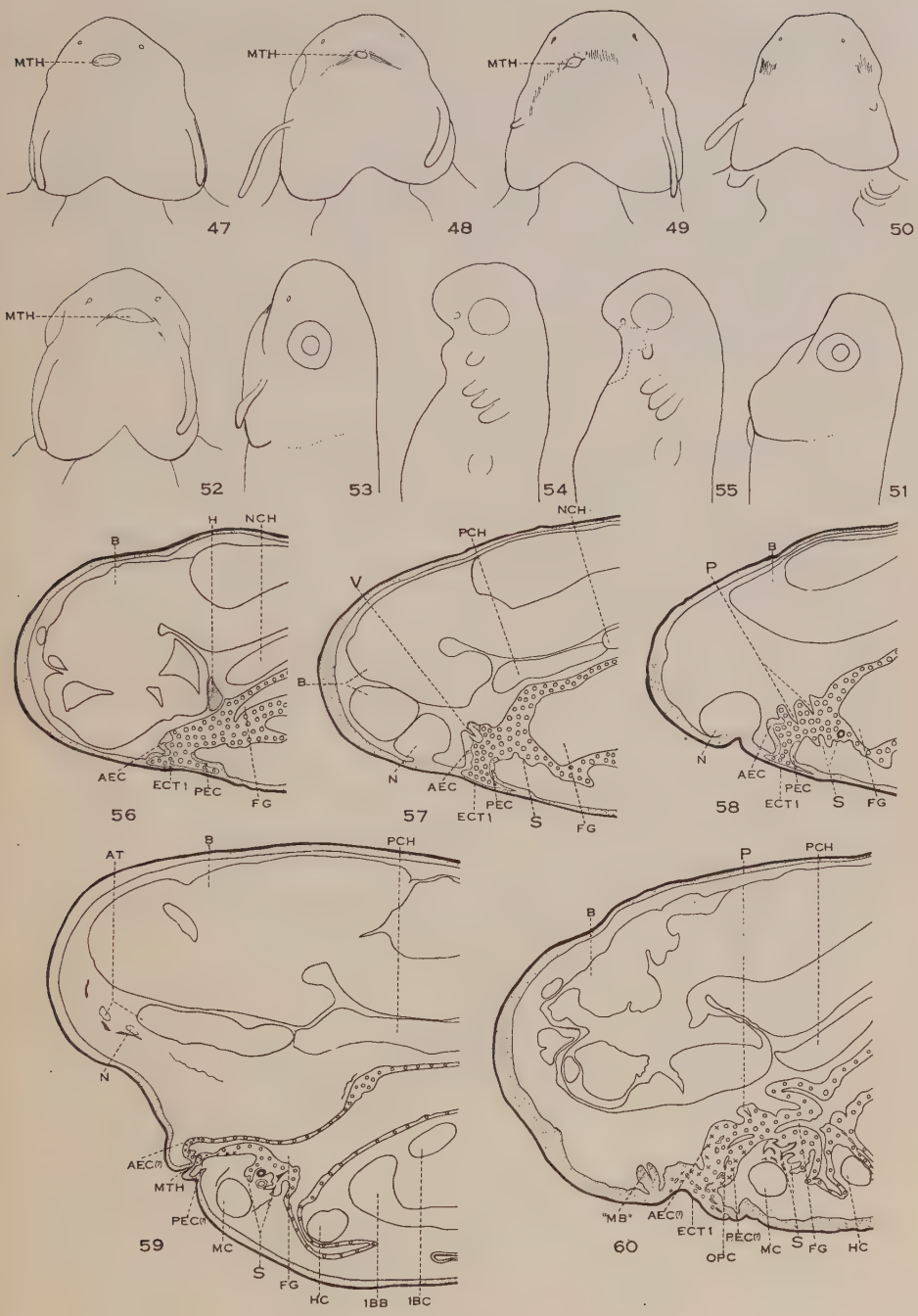
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PLATE 6

EXPLANATION OF FIGURES

61 Sagittal section of specimen StG52, stage 45+, 120 μ to the right of section shown in figure 59, cutting a 'maxillary bulb' containing two tooth germs isolated from the gut cavity. One palatine and three splenial tooth germs are also cut in this section. × 33.

62 Median sagittal section of specimen StG91, stage 42, showing blind end of foregut. The pointed projection at anterior end of gut indicates the separation between dorsal and ventral wall of gut. One palatine and four splenial tooth germs are present in this section. The meckelian cartilage is a solid bar in front of the blind end of the foregut. Two layers of ectoderm cover the ventral mouth region. × 33.

63 Median sagittal section of specimen StG53, stage 45+, in which the foregut is flatly truncated and palatine and splenial tooth germs have their axes parallel to the long axis of the body. Two layers of ectoderm cover the ventral surface of the head. The meckelian cartilage forms a solid bar in front of the blind end of the foregut and is fused to the anterior trabeculae. × 33.

64 Median sagittal section of specimen StG72, stage 40, showing blind end of foregut with procartilage anlage of Meckel's cartilage anterior to it. Four tooth germs of the splenial and palatine(?) sets project into the endodermal tissue. × 33.

65 and 66 Camera-lucida outlines of dissections of visceral cartilages of a normal and an operated animal (StG74), stage 45. × 10.

65 Ventral view of cartilages of normal specimen.

66 Ventral view of operated animal, showing the meckelian cartilages fused into an irregular bar situated in the median line, the hyohyal cartilage(?) pushed forward on the meckelian cartilage and connected with the rest of the hyoid by non-cartilaginous tissue. Only the hyoid and hyohyal(?), first and second branchial cartilages and first and second basibranchials are visible from a ventral view.

67 and 68 Camera outlines of lateral views of visceral cartilages and chondrocranium of normal and operated specimen StG74. × 10.

67 Normal control animal.

68 Operated animal showing abnormal cramping of both chondrocranium and visceral cartilages.

69 and 70 Lateral views of brains of normal and operated specimen StG74, respectively, stage 45. In the operated animal the cerebral hemispheres are small and squeezed more posteriorly than in the normal specimen and the midbrain and cerebellum are pushed back somewhat over the fourth ventricle. The very thin sac-like protrusion of the infundibulum and lack of an hypophysis are quite apparent. × 10.

71 Transverse section of specimen GSt16, stage 45, showing lens which has developed from the stomodaeal ectoderm transplanted to the gill region. × 18.

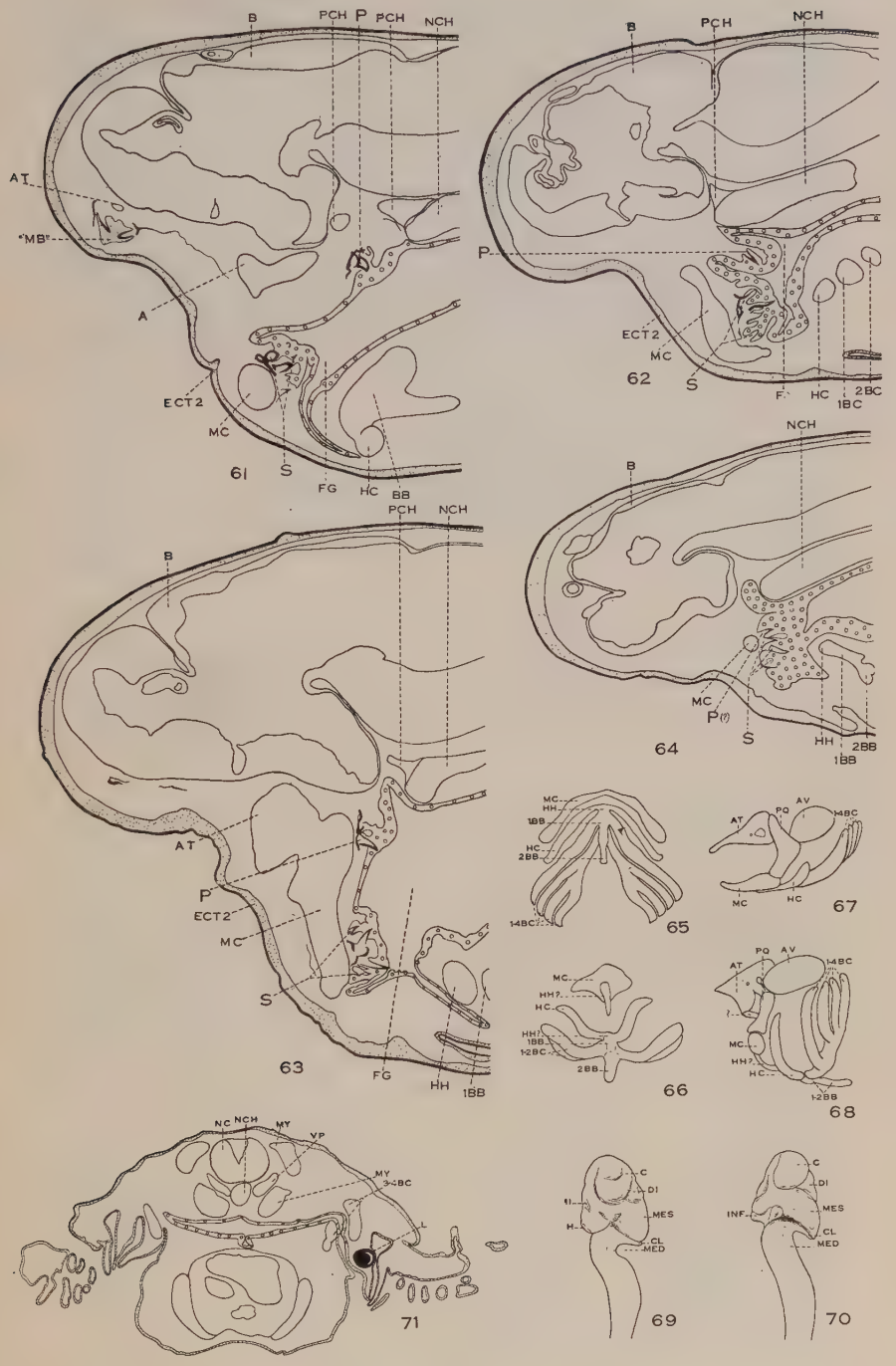
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PLATE 7

EXPLANATION OF FIGURES

72 to 74 Photographs of dorsal, ventral, and lateral views of specimen StG51 (right) and its normal control (left). The small narrow head of the operated specimen and the protruding hyoid and branchial region are very noticeable. × 3.5.

75 Wash drawing of sagittal section through oral plate of embryo in stage 27, showing the oblique position of the cells of the inner layer of ectoderm during the formation of the one-layered component of the oral plate. × 250.

76 Sagittal section through oral plate of stage 29, showing one layer of ectoderm over larger area than in figure 75 and fewer cells in an oblique position. × 250.

77 Part of transverse section of oral plate of stage 29, showing the columnar cells of the inner ectodermal layer forming a distinct border against the endoderm. × 250.

78 to 81 Tooth germs of an embryo, stage 39, showing the characteristic appearance of the enamel organs. × 250.

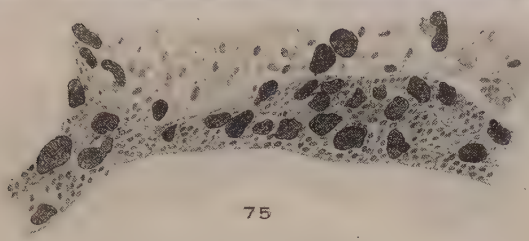
78 Section of the ectodermal-endodermal enamel organ of a median dentary tooth.

79 Section of a vomerine tooth entirely within the ectodermal collar.

80 Section of a vomerine tooth germ on the border between ectodermal collar and endoderm. Only a small part of the base of the cap contains large yolk granules. The remaining sections of the tooth germ indicate that the enamel organ is largely ectodermal.

81 Section of a palatine tooth germ, showing large yolk granules in the endoderm of the enamel organ. The ectodermal cells of the collar may be seen jutting against the endodermal layer. A section of a splenial tooth germ resembles that of a palatine germ except that the ectodermal collar does not come in contact with the former.

82 Sagittal section of specimen St23a, stage 25, showing the projections from cells of the external ectodermal layer in the process of wound healing. × 250.



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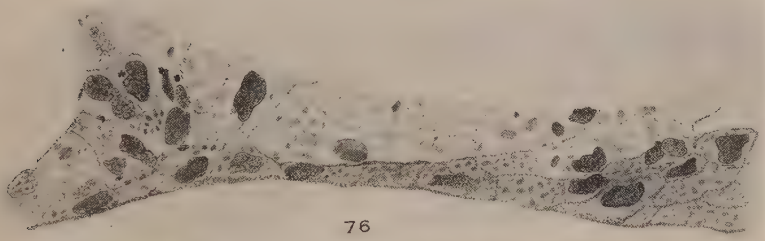

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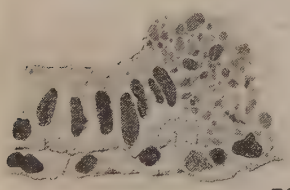

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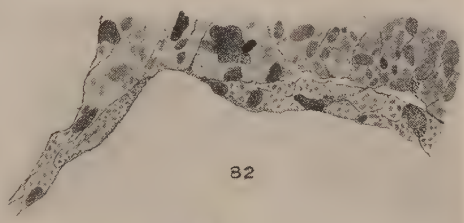

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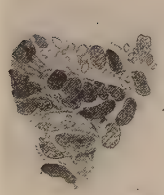

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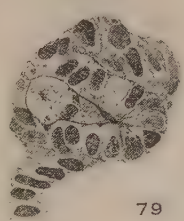

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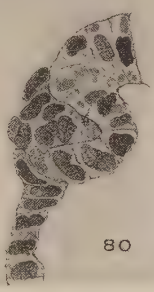

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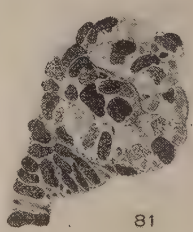

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IS THE TADPOLE TEST FOR ~~TYPHOID~~ Thyroid VALID?

W. W. SWINGLE,
Osborn Zoological Laboratory, Yale University,
and the Biological Laboratory,
Cold Spring Harbor

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IS THE TADPOLE TEST FOR THYROID VALID?

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It is a matter of common observation to clinicians and experimentalists that various samples of commercial thyroid preparations may differ considerably with regard to their physiological activity. The value of this gland as a therapeutic agent in the treatment of hypothyroid cases, has of late years been considerably increased by the adoption of a method of standardizing different preparations on the basis of their iodine content.

However, it requires but little reflection to see that such a chemical method is not entirely satisfactory for determining the physiological effectiveness of thyroid preparations. Only a certain special kind of iodine complex is active, and to date no other type of iodine compound, inorganic or organic, is known that is capable of simulating in a qualitative or quantitative manner the effects produced upon mammalian metabolism, and on myxedema and cretinism, by the idoine complex peculiar to the thyroid gland, i. e., thyroid iodine. Consequently, the iodine content of thyroid preparations should refer only to thyroid iodine, but unfortunately such is not the case. A large part of the iodine contained in the thyroid is said to exist in inactive form and is apparently united with amino acid complexes, possibly representing intermediate steps in the synthesis of the active hormone.

It has been shown (1) that hyperplastic thyroids extremely low in iodine content are capable of absorbing large amounts of iodine both in vivo and in vitro, and when potassium iodide is injected into the circulation of an animal with goitrous or hyperplastic glands the iodine is absorbed by the thyroid practically as soon as it reaches the gland. Such thyroids when assayed can be made to show any per cent of iodine. But despite the high iodine content such glandular tissue has little thera-

peutic value, because the iodine present is not thyroid iodine, i. e., in thyroxin form. A certain time is required for the thyroid to convert the iodine it receives into the combination peculiar to its hormone.

Since Gudernatsch's discovery of the remarkable effect of thyroid upon amphibian metamorphosis, several investigators have suggested that this reaction be employed as a method of physiological assay for testing the activity of thyroid preparations, as a supplement or even substitute for the present method of standardization by means of the iodine content.

Romeis (2) was the first to propose the use of the tadpole test as a means for standardizing commercial thyroid and iodothyrine preparations. He observed great variations in the activity of the preparations tested, as manifested by the rapidity with which the material induced metamorphic change in frog larvae when fed, and stated that his results with amphibian larvae agreed with similar experiments on dogs, and with clinical observations. He did not compare the activity of the different preparations with their iodine content. Lenhart (3) showed that desiccated thyroid when fed to tadpoles induces effects on their growth and differentiation in proportion to the quantity administered and the amount of iodine present. He suggested that the tadpole reaction to thyroid might serve as a biological test for the activity of thyroid preparations, superior even to chemical methods.

Rogoff (4) made use of the action of thyroid upon amphibian metamorphosis to assay the physiological value of various commercial thyroid preparations. In his paper he advances cogent reasons why the present method of standardization of thyroid by determination of its iodine content is not an entirely reliable indication of its therapeutic value, and suggested the tadpole test as a method of physiological assay.

Jensen (5) proposed the use of the axolotl for the standardization of thyroid preparations. As is well known, this animal is the paedogenetic larvae of Amblystoma tigrinum, and readily responds to thyroid administration by metamorphosis. Various commercial extracts of thyroid and iodothyrine were tested on

axolotls and it was found that the efficiency of the preparations used was not directly proportioned to their iodine content.

Hogben and Crew (6) employed the axolotl as a means of determining the degree of physiological activity of fetal and embryonic ox and sheep thyroids. A single meal of fetal thyroid was fed to axolotls, and if metamorphic changes were initiated it was regarded as demonstrating the fact that such glands were actually building up their active products.

It is quite evident that the first four authors mentioned regard the present method of standardizing thyroid preparations by their iodine content as unsatisfactory, but it is questionable whether the suggested utilization of the metamorphic changes of amphibian larvae as a method of physiological assay of thyroid activity is likely to prove any more satisfactory. In fact the writer is inclined to doubt if the tadpole test is really valid, and believes that it is really a test for a peculiar type of organic iodine, and not specific for thyroid iodine. The following experiments invalidate the tadpole test for thyroid activity and preclude the employment of these animals for physiological assays of thyroid products (7).

The three blood proteins, serumalbumin, serumglobulin and fibrin, have little if any effect upon the differentiation of thyroidectomized and hypophysectomized frog tadpoles. However, if these substances are iodized properly they become highly active metamorphosis-inducing agents when fed or injected. The addition of the iodine to the molecule of these proteins transforms an inactive substance, insofar as frog metamorphosis is concerned, into an agent which simulates the thyroid hormone (although the acceleration of metamorphosis is not so rapid) in the effect produced upon anuran differentiation. Iodized casein behaves in a similar fashion, although this protein does not appear to be quite so active as the others mentioned. Iodogliadin if fed in large quantities for two months or more will metamorphose hypophysectomized tadpoles. The metamorphosis-inducing activity of this vegetable protein is distinctly less than the other iodized proteins tested. However, the activity of any one of these proteins depends upon the method of iodization. The looser the combination effected between the iodine and the protein, the less the activity upon differentiation. In

contrast to iodogliadin, the vegetable protein edestin is an active metamorphosis inducing agent when properly iodized, although apparently not so active as the blood proteins mentioned. Rogoff (8) states that iodized egg albumin (fresh egg white) shows some activity when fed to normal tadpoles, but that the activity is not very great.

The writer has not found iodalbin (Parke, Davis & Co.) nor iodocasein (Mulford & Co.) very active in inducing metamorphic change. In both of these commercial preparations of iodized protein the iodine is in loose combination and is very easily split off the protein molecule; some specimens seem to be more active than others.

The structure of the protein molecule is as yet unknown; consequently the nature of the iodine linkage is undetermined. However, one of the amino acids, tyrosine, has been successfully iodized and is known to be an effective agent in inducing the metamorphosis of both thyroidless and hypophysectomized tadpoles. Tyrosine is inactive, dibromotyrosine is inert, but diiodotyrosine is very effective in acceleratng transformation. It is clear from the experiments upon iodotyrosine that the effective agent in amphibian transformation is the iodine in the molecule, bromine cannot be substituted for it and the tyrosine remain active. Moreover, the nature of the iodine linkage in diiodotyrosine is known and this amino acid is not chemically related to thyroxin, the active principal of the thyroid. Perhaps even more interesting in this connection is the fact that tyramine, the decarboxylated product of tyrosine, when iodized, is an active agent in producing metamorphosis (9). The iodine linkage in tyramine is the same as in tyrosine, i. e., to the 3rd and 5th carbon atoms of the benzene ring.

Investigators who have worked upon this phase of the problem agree that iodized proteins such as iodoserumglobulin, albumin and casein show little if any thyroid-like effects upon mammalian metabolism, nor does the administration of such substances alleviate the symptoms of myxedema or cretinism.

Oswald (10) studied the physiological properties of several iodized proteins and compared them with thyroid. He found that iodocasein, iodoserumalbumin and diiodotyrosin do not exert thyroid-like effects when administered to mammals.

Hellin (11) reported that iodoalbumin and nucleo-albumin prepared from the spleen are inactive when given to mammals, and Blum (12) states that iodoalbumin has practically no effect upon metabolism.

Strouse and Voegtlin (13) did not observe any thyroid-like effects upon the nitrogen metabolism or on the blood pressure of normal dogs, nor was there any favorable effect on the condition of myxedematous and cretinous mammals following administration of diiodotyrosin.

It is evident that the substances which accelerate metamorphic change in amphibians, thus simulating the activity of the thyroid hormone in this vertebrate group, do not exert thyroid-like effects when administered to mammals, at any rate insofar as metabolism is concerned. Perhaps the most convincing evidence that the effect of thyroid upon amphibian metamorphosis and upon mammalian metabolism is due to different chemical groupings in the thyroid hormone is that adduced by Kendall (14) and the writer (15) on the acetyl derivative of thyroxin. The following experiment was recently performed by the writer. Two normal, healthy adults took 10 milligrams of crystalline thyroxin intravenously; a profound physiologic response, comparable in every way to that evoked by excessive thyroid feeding, was evoked, the effect lasting about six days. Several weeks later the same two subjects received an intravenous injection of 10 mg. of thyroxin in which the hydrogen of the imino group had been replaced by acetyl. The injection produced no response whatever. Kendall earlier had noted that acetyl thyroxin when injected into dogs produced no physiologic response, whereas an equal amount of thyroxin did produce a profound response lasting some time. The fact that when the hydrogen of the imino group in thyroxin is replaced by acetyl the substance loses its physiologic activity and there follows no demonstrable effect upon mammals emphasizes the importance of the imino group and minimizes the importance of iodine in the molecule insofar as the physiologic effect upon mammals is concerned. However, both thyroxin and the acetyl derivative are highly active agents in inducing metamorphosis of thyroidless and hypophysectomized amphibians. The con-

clusion seems justified that the physiologic effect of thyroxin and thyroid in mammals is due to the CONH group within the molecule, because when the hydrogen of this group is replaced by acetyl the thyroxin becomes inert, yet the iodine complex remains unchanged. On the other hand, it is the iodine complex in the molecule which is effective in accelerating amphibian metamorphosis. The reasons for this conclusion are obvious: (1) the acetyl derivative of thyroxin is highly effective despite the change in the CONH group which is rendered inactive; (2) iodotyrosin is highly effective in inducing amphibian metamorphosis and there is no CONH group in this substance; (3) certain iodized proteins which are inactive when administered to mammals are highly active in metamorphosing amphibians.

Now what is the bearing of this evidence upon the validity of the tadpole test for thyroid? Simply this, that the tadpole test is a reaction to a certain type of organic iodine complex found in the thyroid and gives no clue to the activity of or even presence of other chemical groupings within the molecule, for instance the CONH group, which alone (though probably in conjunction with iodine) has an effect upon mammalian metabolism and upon myxedema and cretinism. Acetyl thyroxin gives a positive tadpole reaction, but this substance has no effect upon myxedema or cretinism.

It is evident that the use of tadpoles for testing the physiological activity of thyroid preparations is unsatisfactory. Thyroid samples, giving a very positive test when fed to thyroidectomized tadpoles, might be totally inert when fed to mammals insofar as the metabolic rate and the alleviation of myxedema and cretinism are concerned.

It is unjustifiable to assume that the iodine containing complex responsible for amphibian transformation is identical with that which has to do with mammalian metabolism. Iodine seems essential to both chemical groups, i. e., the one responsible for mammalian metabolism and the group responsible for amphibian metamorphosis, but in mammals other constituents of the molecule such as the CONH complex are of equal or even greater importance.

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14. Kendall: Am. J. Physiol. (Balt.), 1919, **49**, 136.
15. Swingle: Am. J. Physiol. (Balt.), 1924 (in press).

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STUDIES OF A PEDIGREE CULTURE OF PARAMECIUM CALKINSI

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FOUR FIGURES

Refinements in cultural methods have gradually prolonged the time during which ciliates will live without conjugation in the laboratory, until it appears to be positively established that unsuitable environment is the factor which previously led to the view that death is inevitable when conjugation is prevented.[1] However, the study of the life-history of Paramecium aurelia and Paramecium caudatum has shown that the division rate is punctuated by periodic fluctuations, termed rhythms, which uniform conditions fail to remove.[2] With the discovery of endomixis at the low point in the rhythms in Paramecium aurelia and Paramecium caudatum, the cause of the rhythms seemed established;[3] but the fact that endomixis has never been described during the free life of certain ciliates, in particular hypotrichs, which apparently can live indefinitely without conjugation although they exhibit rhyhms seemingly comparable with those of Paramecium, suggests that this nuclear reorganization phenomenon, as

[1] Woodruff, L. L., The present status of the long-continued culture of Paramecium aurelia at Yale University, Proc. Nat. Acad. of Sciences, vol. 7, 1921; Woodruff, L. L., and Spencer, H., Studies on Spathidium spathula. II. The significance of conjugation, Jour. Exp. Zoöl., vol. 39, 1924; Woodruff, L. L., and Moore, E. L., On the longevity of Spathidium spathula without endomixis or conjugation, Proc. Nat. Acad. of Sciences, vol. 10, 1924.

[2] Woodruff, L. L., and Baitsell, G. A., Rhythms in the reproductive activity of Infusoria, Jour. Exp. Zoöl., vol. 9, 1911.

[3] Woodruff, L. L., and Erdmann, R., A normal periodic reorganization process without cell fusion in Paramecium, Jour. Exp. Zoöl., vol. 17, 1914.

well as conjugation, may not be a necessity for the continued life of the cell.[4]

Paramecium calkinsi was found to be favorable material for the study of this problem, and accordingly experiments were inaugurated in an attempt to answer the questions: 1) Can Paramecium calkinsi live indefinitely without conjugation or endomixis? 2) Are rhythms evident without endomixis?

The author wishes to express to Prof. L. L. Woodruff her appreciation of his constructive criticism extending through the duration of the work.

HISTORY OF THE CULTURES

The animal used to start culture I of these experiments was taken on May 26, 1921, from the original Paramecium calkinsi

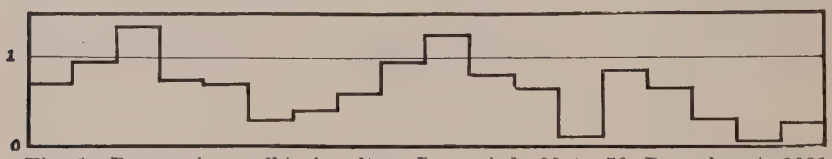

Fig. 1 Paramecium calkinsi, culture I: periods 39 to 56, December 4, 1921, to March 3, 1922, culture kept at room temperature. Graph showing average division rate of four lines again averaged for five days.

culture[5] and bred, as were all the pedigree cultures, on standard beef extract.[6] Isolations were, in general, made at the same time daily, and the bacterial flora was kept the same, it is believed, by daily cross-infection. Such external factors as daylight and barometric pressure need not be considered, since they have already been shown to be without significant effect on the colorless Paramecia.[7] Culture I was carried at room temperature until July 21, 1922, when it was stopped, after being in pedigree culture 420 days and attaining 392

[4] Woodruff, L. L., An experimental study of the life-history of hypotrichous Infusoria, Jour. Exp. Zoöl., vol. 2, 1905; Woodruff, L. L., and Spencer, H., loc. cit.; Moore, E. L., loc. cit.

[5] Woodruff, L. L., The structure, life history, and intrageneric relationships of Paramecium calkinsi, sp. nov., Biol. Bull., vol. 41, 1921.

[6] Woodruff, L. L., and Baitsell, G. A., The reproduction of Paramecium aurelia in a 'constant' culture medium of beef extract, Jour. Exp. Zoöl., vol. 11, 1911.

[7] Woodruff, L. L., and Baitsell, G. A., Jour. Exp. Zoöl., vol. 9, 1911, loc. cit.

generations. Figure 1 shows a small but typical part of the graph of the division rate.

Culture II was started on September 13, 1921, as a subculture of culture I at the 171st generation. The animals were kept in a Freas thermostat at a practically constant temperature. Food, pipets for the isolation of the indi-

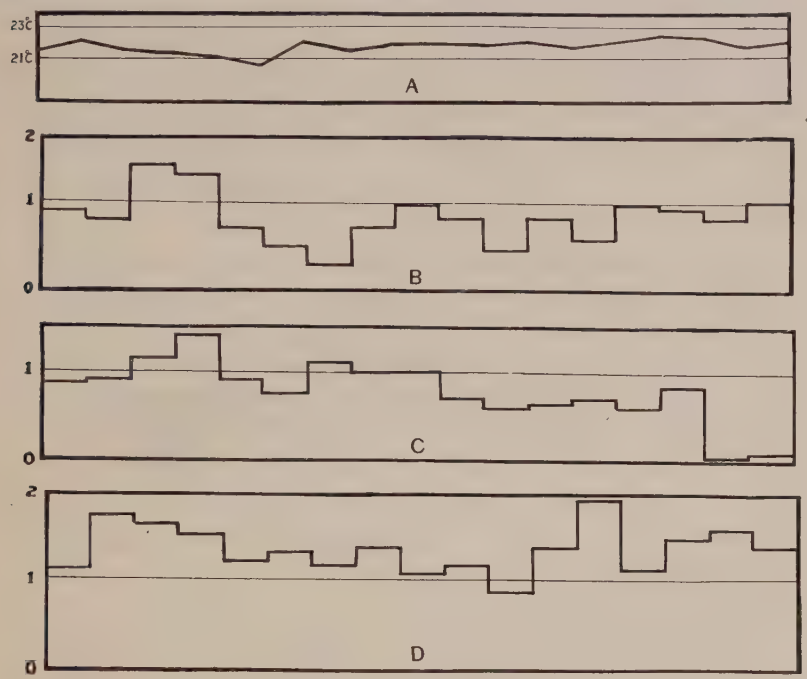

Fig. 2 A, the average daily temperature again averaged for five days. B, Paramecium calkinsi, culture II. C, Paramecium calkinsi, culture III. D, Paramecium aurelia. All cultures kept at the same temperature and under 'constant' conditions during periods 39 to 56, December 4, 1921, to March 3, 1922. Method of plotting the same as in figure 1.

viduals, and slides to which they were transferred were also kept in the same thermostat further to minimize changes in temperature. On June 11, 1922, culture II died at the 420th generation, having lived synchronously with culture I for 272 days (fig. 2, B).

On September 20, 1921, culture III was isolated from stock of the original culture in hay infusion, and was bred on beef extract until it died on February 25, 1922, having lived 154

days and attained 145 generations. The environmental conditions of this culture were the same as those for culture II; that is, the animals were kept in the same thermostat and bred on the same supply of beef (fig. 2, C).

Culture IV was started as a subculture from culture II on April 29, 1922. These animals were bred to determine the influence of different environmental conditions on the fluctuations in the division rate. Accordingly, the culture was carried in another room where the temperature was not the same and a different bacterial flora was supplied, although the basic medium, standard beef extract, was used as before. These lines were stopped after 80 days, when they had reached 54 generations (fig. 3).

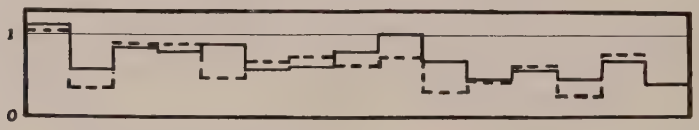

Fig. 3 Paramecium calkinsi, culture IV (– – –), periods 62 to 76, March 30, 1922, to June 11, 1922, using culture II (——) for comparison during synchronous periods. Conditions diverse.

For comparison with P. calkinsi cultures, two cultures (V and VI) were started from Woodruff's original Paramecium aurelia culture on September 15, 1921, and carried in the Freas thermostat until the end of the definitive experiments on July 21, 1922. Endomixis was readily demonstrated soon after the cultures were under observation, thereby showing that the reorganization occurred as usual at the low points in the rhythms in Paramecium aurelia (fig. 2, D).

CONJUGATION

In an attempt to induce conjugation in this race of P. calkinsi stock flasks of the animals in hay infusion have been maintained throughout the entire history of the culture and up to the present time—May, 1924. Material from the top, middle, and bottom of the flasks was examined practically daily for a period of about two months. Further observations were made at varying intervals during the rest of the more

than three years over which this study has extended. Not a single pair of conjugants was seen, and accordingly it may be accepted with practical surety that conjugation did not occur.

Since Zweibaum[8] stated that conjugation was most readily effected by allowing the animals to starve for some weeks, and then treating them with a salt, notably with N/50000 $FeCl_3$ and N/1200 NaCl, the same procedure was followed with P. calkinsi. In some cases these solutions were alone used as media, in others in combination with beef or hay. Both starved and well-fed animals were employed. Not a single pair of conjugants resulted.

In experimental work on Paramecium, Hopkins[9] found that N/1000 $NaNO_3$ aided in bringing about conjugation. Accordingly, animals, both well-fed and starved, were subjected to varying strengths of this salt in numerous combinations with hay and beef. These conditions failed to produce conjugants.

Jennings[10] stated that old, starved cultures, if revived with fresh food until they are in a heathy condition and then suddenly starved, will frequently provide epidemics of conjugation. This procedure was carried out in detail and also with certain modifications. Sometimes animals which had been starved in beef were made normal again by a sudden change to hay infusion and vice versa. Rapid starvation was accomplished either by changing the culture to an old medium, the same kind in which it had been living, or to another, or by putting the animals into distilled water. Slow starvation was realized by allowing a heavy culture to exhaust all the available food or by putting the cultures on ice so that the bacterial food supply could not develop so readily. Furthermore, heavy cultures were allowed to evaporate at different rates. All these methods proved of no avail; conjugation did not occur.

[8] Zweibaum, J., Les conditions nécessaires et suffisantes pour la conjugason du Paramecium caudatum. Arch. f. Protistenk., Bd. 26, 1912.

[9] Hopkins, H. S., The conditions for conjugation in diverse races of Paramecium. Jour. Exp. Zoöl., vol. 34, 1921.

[10] Jennings, H. S., What conditions induce conjugation in Paramecium. Jour. Exp. Zoöl., vol. 9, 1910.

Since Woodruff's Paramecium aurelia culture was under observation for about six years before conjugation was observed,[11] it is inadvisable definitely to conclude that the phenomenon cannot occur in this race of P. calkinsi, but it is certain that conjugation can be dispensed with for long periods of time without injury to the normal processes of the cell, as indicated by the usual criterion—the division rate. The lack of conjugation is remarkable, since the ciliates already studied, e.g., Spathidium,[12] which do not exhibit endomixis in the active phase of their life-history, usually conjugate frequently.

ENDOMIXIS

An analysis of the cytological data accumulated during the study of the cultures was made with special reference to endomixis. As briefly reported by Woodruff, about 1000 preserved specimens of P. calkinsi, taken at various points in the life-history, were examined for evidence of endomixis. During a period of about a month, nearly every sister cell of the main line was stained and studied cytologically. Thus, the possibility of failing to find endomixis, were the phenomenon present, was practically precluded, but no evidence of endomixis was found.[13]

In the present series of experiments, over 200 preserved animals were examined. These were killed chiefly at the low points in the division rate, where endomixis in P. aurelia has been shown to occur regularly, but in no case have there been departures from the nuclear structure typical of the vegetative animals. It can therefore be definitely reaffirmed that endomixis does not occur in P. calkinsi during active life.

In some forms, notably Didinium nasutum, Uroleptus mobilis, and Spathidium spathula,[14] endomixis has been found

[11] Woodruff, L. L., On so-called conjugating and non-conjugating races of Paramecium. Jour. Exp. Zoöl., vol. 16, 1914.

[12] Woodruff, L. L., and Spencer, H., loc. cit.

[13] Woodruff, L. L., Biol. Bull., loc. cit.

[14] Calkins, G. N., Didinium nasutum. I. The life history, Jour. Exp. Zoöl., vol. 19, 1915; Uroleptus mobilis Engelm. II. Renewal of vitality through conjugation, Jour. Exp. Zoöl., vol. 29, 1919; Moore, E. L., loc. cit.

to occur solely during encystment. This raises the question whether P. calkinsi encysts and, if so, whether endomixis occurs during encystment.

A few cases of encystment in Paramecium of different species have been recorded by Prowazek, Simpson, and others,[15] but these observations have not been corroborated. Indeed, numerous intensive studies on P. aurelia and P. caudatum have failed to show positive examples of encystment, and it may be accepted that encystment does not occur. Likewise, in the present experiments on P. calkinsi, careful study of animals under many kinds of environmental conditions has not revealed a single cyst. Thus, there is no evidence of encystment in P. calkinsi, and therefore endomixis obviously is precluded from this stage in the life-history. Accordingly, it may be concluded that endomixis does not occur at all in the life-history of P. calkinsi.

RHYTHMS

Although endomixis is not present in this race of P. calkinsi, the graphs of these cultures show that fluctuations in the division rate occur. In order to determine whether these fluctuations, in the absence of endomixis, are due to extrinsic or intrinsic factors, a comparison of the several cultures was made.

A study of the graphs of the P. calkinsi cultures reveals the fact that fluctuations in the rate of reproduction are present which apparently are indistinguishable from the rhythms of P. aurelia and P. caudatum. Since the environmental conditions of the cultures compared were as near identical as is possible under the conditions of the experiment, there was afforded a favorable opportunity for synchronous rhythms to occur, if these fluctuations are fundamentally extrinsic in origin. From figures 1 and 2 it will be seen that the low points in the fluctuations of the division rate do not occur

[15] Prowazek, S., Kleine Protozoenbeobachtungen, Zool. Anzeiger, Bd. 22, 1899; Simpson, J. Y., Studies on Protozoa; notes on the intimate structure of Protozoa, as exhibited by intravital staining, Proc. Scot. Mic. Soc., vol. 3, 1901.

synchronously with any degree of regularity in the various cultures being carried simultaneously, and this fact is equally evident throughout the whole history of the cultures. There is a similar lack of coincidence in the high points in the division rate. Temperature is the environmental factor to which one would naturally turn for the cause of the fluctuations in the division rate, but it will be noted that in the graph the temperature shows very slight variation for the entire latter half, yet the variations in the division rate of the several cultures during this time indicate the usual fluctuations. All the data, therefore, seem to show that external conditions do not cause rhythms.

DISCUSSION

From a consideration of the foregoing experiments, the conclusion is patent that conjugation is not necessary for the continued life of this race of P. calkinsi, as already proved for P. aurelia. Of the pedigree cultures, only one, culture I, was alive at the termination of the definitive experiments, but of the others, culture IV had been stopped just previously, and cultures II and III had died. The pedigree culture of P. calkinsi carried by Woodruff had also died. However, at any time during the experiments the animals were readily cultured in small mass cultures in hay infusion. These hay-infusion cultures, carefully watched to preclude the possibility of conjugation occurring unnoticed, are still under observation and dividing actively. This fact renders it impossible to interpret the death of the slide cultures as a result of degeneration, which is implied in the conception of a cycle, but rather indicates that the medium employed, known to be highly suitable for P. aurelia, is, however, not perfectly favorable for P. calkinsi. Accordingly, no evidence is afforded by this study of an inherent 'cycle' in the life-history of P. calkinsi which terminates in death if conjugation is prevented.

Equally convincing is the evidence that endomixis does not occur at any period in the life-history of P. calkinsi, and

accordingly the conclusion is unavoidable that neither conjugation nor endomixis is indispensable for the indefinite continuance of reproduction by division in this species of Paramecium.

But although endomixis does not occur, there are periodic fluctuations in the division rate which are not distinguishable from those described in various infusoria by Woodruff[16] and defined as a "minor rise and fall in the fission rate due to some unknown factor in cell metabolism from which recovery is autotomous." Later, Woodruff and Erdmann, when they discovered endomixis, believed that this nuclear reorganization was the unknown factor responsible for the rhythms. Undoubtedly in P. aurelia and P. caudatum endomixis is synchronous with the low points of the rhythms, but that endomixis is solely responsible for rhythms is improbable in view of these results with P. calkinsi. In this species, which does not exhibit endomixis, fluctuations occur which would seem to be rhythms, in the sense that term was employed by Woodruff in 1905.

SUMMARY

1. Conjugation has not occurred during four years that Paramecium calkinsi has been under observation, and all attempts to induce it have failed.

2. Endomixis does not occur in this race of P. calkinsi and the race can live indefinitely without this process.

3. Encystment does not occur in this species.

4. Since neither endomixis nor conjugation has occurred in P. calkinsi under laboratory conditions and the animal shows every indication of the potentiality to reproduce by division indefinitely, it is clear that neither phenomenon is necessary for the successful continuation of life.

5. Fluctuations in the division rate, which are regarded as rhythms, occur, although endomixis is absent. Constant conditions do not eliminate them, and therefore rhythms are due to some factor inherent in the organism other than endomixis.

[16] Woodruff, L. L., Jour. Exp. Zoöl., 1905, loc. cit.

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